Biological properties and clinical relevance of disseminated tumour cells in patients with hepatocellular carcinoma

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BIOLOGICAL PROPERTIES AND CLINICAL RELEVANCE OF DISSEMINATED TUMOUR CELLS IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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by

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Abstract:

Background: Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is the third leading cause of cancer related deaths worldwide. Surgical treatments provide a 5 year survival of approximately 60%-70% in carefully selected patients. However tumour recurrence to the liver continues to be a significant issue. Disseminated tumour cells (DTC) have been associated with tumour recurrence and poor prognosis in several epithelial cancers. The biological properties of DTC in HCC and their association with adverse clinical outcomes have not been critically investigated.

Methods: DTC were cultured from peripheral blood samples using a protocol that was developed in house. Their molecular and biological properties were studied using immunocytochemistry, molecular assays and a SCID mouse cell transplantation model.

Results: Venous blood was collected from 44 patients with HCC, 50 with liver cirrhosis and 20 healthy volunteers. Following 2 weeks of *in vitro* culture, cell colonies were observed in 6 patients with HCC and 3 patients with cirrhosis. No cell growth was noted in blood cultured from healthy volunteers. Immunocytochemical analysis of cultured cells showed that they express characteristic markers of human hepatocytes. Further they also expressed phenotypic markers compatible with bone marrow or cancer stem cells. Cells cultured from patients with HCC exhibited increased expression of glypican-3 and survivin and decreased expression of LYVE1. They also demonstrated 18- and 43-gene expression signatures associated with poor prognosis in HCC and non-HCC solid tumours. DTC from HCC patients persisted in SCID mice and showed histological features of neoplasia. Survival analysis showed that HCC patients with DTC had reduced median survival compared to those with no DTC (13 months vs. 49 months).

Conclusion: DTC are present in a sub-group of patients with aggressive tumour phenotype. An in depth knowledge of these cells is essential to develop novel cell targeted therapies to eradicate microscopic disease and improve survival.

KEYWORDS:

Hepatocellular carcinoma

Disseminated tumour cells

Micrometastases

Circulating tumour cells

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LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
BCLC	Barcelona Clinic Liver Cancer
BMDC	Bone marrow derived cells
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
C-GSF	Granulocyte colony-stimulating factor
CLIP	Cancer of the liver Italian programme
CSC	Cancer stem cells
CT	Computed tomography
DAB	Diaminobenzidine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTC	Disseminated tumour cells
ECACC	European collection of cell cultures
ECGS	Endothelial cell growth supplement
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Essential modified eagle medium
FCS	Fetal calf serum
H&E	Haematoxylin and Eosin
HBV	Hepatitis B virus

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
Hep Par-1	Hepatocyte paraffin-1 mouse monoclonal antibody
HGF	Hepatocyte growth factor
HPC	Hepatic progenitor cell
HRP	Horseradish peroxidase
HSA	Hepatocyte specific antigen
HSC	Haematopoietic stem cell
IGFR	Insulin-like growth factor
LT	Liver transplantation
MCT	Microwave coagulation therapy
MELD	Model for End-Stage Liver Disease
MEM	Minimal Essential Medium
MET	Mesenchymal epithelial transition factor
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
N-2 AAF	N-2-acetylaminofluorene
NASH	Non-alcoholic steatohepatitis
OLT	Orthotopic liver transplant
PAI	Percutaneous acetic acid injection
PBS	Phosphate buffered saline

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PEI	Percutaneous ethanol injection
PET-CT	Positron emission tomography combined with computed tomography
PI	Propidium iodide
RFA	Radio-frequency ablation
RNA	Ribonucleic acid
RNOS	Nitrogen oxide free radicles
Rpm	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor-1
TACE	Trans-arterial chemo-embolization
TGF α	Transforming growth factor-alpha
TNF α	Tumour necrosis factor-alpha
TNM	Tumour Node Metastasis classification system
UCSF	University of California, San Francisco

USS	Ultrasonography
UW	University of Wisconsin
VEGF	Vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1.1 Hepatocellular carcinoma (HCC) is the most common primary liver tumour, the sixth most common malignancy worldwide and the third most common cause of cancer related deaths (Parkin 2005). There is an uneven distribution in its incidence and marked variation in the underlying risk factors according to the region. The majority of HCC cases (80%) occur in sub-Saharan Africa or Eastern Asia. The incidence of HCC in the UK is approximately 2.2/100,000 patients. While hepatitis B virus (HBV) infection is the main risk factor in Asia and Africa, hepatitis C virus (HCV) is a significant problem in Western countries and Japan. The exception to this observation is immigrant population to the West in whom HBV infection is still the predominant aetiology for HCC. Other reported risk factors include exposure to Aflatoxin, a mycotoxin produced by the Aspergillus fungus, heavy alcohol intake (more than 50–70 g/day) for prolonged periods, hereditary haemochromatosis, metabolic syndrome and insulin resistance. In recent years there has been increasing evidence to support an association between obesity and HCC. Obesity is associated with diabetes mellitus and non-alcoholic steatohepatitis (NASH), conditions that are now believed to increase the risk of HCC (Starley 2010). NASH is estimated to be the third most common liver disorder in North America and the most common in Australia and New Zealand. It is estimated that NASH will progress to liver cirrhosis in nearly 25% of patients. There has been a significant increase in the incidence of HCC in the West due to an increase in hepatitis C infection and alcohol intake. HCC is currently the leading cause of death among patients with cirrhosis (Sangiovanni 2004). The pathophysiology of hepatocarcinogenesis is linked to the evolution of cirrhosis which develops after long latencies of chronic liver disease. The cirrhotic liver is a preneoplastic condition and is the strongest predisposing factor for developing HCC, however, in 10% of cases HCC occurs in a normal liver.

The molecular pathogenesis of HCC is complex, the progression of chronic inflammation to cirrhosis and HCC may last 10-30 years (Buendia 2000, Thorgeirsson 2002). Several mechanisms are involved in the pathogenesis of HCC depending on the underlying aetiology. The whole process can be broadly summarised into the following steps; 1) Cirrhosis associated with hepatic regeneration and scarring after chronic tissue injury 2) mutations in a single or multiple oncogenes or tumour suppressor genes stimulating cellular proliferation (El-Serag 2007).

Hepatitis viruses: HBV and HCV induce liver injury, hepatocyte death and promote hepatocarcinogenesis but the mechanisms are different. HCV infection results in up regulation of pro-inflammatory, pro-apoptotic and pro-proliferative genes. HCV may act as a Wnt ligand, and it has also been implicated in RAS transactivation and p53 inactivation (Llovet 2009). The role of HBV in HCC carcinogenesis has been well established. HBV DNA integrates into the host genome and promotes expression of cellular genes that are important for cell growth and differentiation. HBV also induces genomic instability, genetic aberrations, inactivation of tumour suppressor genes and assists the production of oncoproteins. Some of these gene products especially HBV X protein (HBx) has been strongly implicated in liver carcinogenesis (Peng 2005).

Chemical injury and Genetic predispositions: Aflatoxin B is a very potent mutagen and its active metabolite reacts with guanine in DNA leading to mutations. In geographical areas of aflatoxin B1 dietary exposure, such as China and Africa, there is a strong association between mutations in P53 (at codon 249) and exposure to this agent (Hussain 2007). Prolonged exposure to chemicals or hereditary and metabolic disorders causes chronic inflammation which results in the release of free radicals, chemokines and cytokines. These reactive oxygen and nitrogen oxide free radicles (RNOS) along with their reaction products causes DNA damage. Further RNOS enhances the enzymatic activity of cyclooxygenase-2 which results in activation of the WNT pathway contributing to HCC carcinogenesis (Grisham 2002).

Genetic aberrations and signalling pathways in liver carcinogenesis: Several genes and cytogenetic aberrations have been described in HCC. The most frequently deleted chromosome arms are 17p, 8p, 16q, 16p, 4q,9p, 13q, 1p and 6q, whereas the most frequent chromosomal gains are located on 1q, 7q, 8q and 17q. Mutations in p53 gene are noted in 30% and telomere length is altered in more than 80% of human HCCs.

Abnormal activation of cell signalling pathways results in a neoplasm. The critical signalling cascades that perpetuate liver cancer are; 1) WNT-β-catenin signalling pathway which is the most commonly disrupted pathway in HCC. Abnormal regulation of transcription factor β-catenin results in expression of gene products involved in cell proliferation angiogenesis, anti-apoptosis and the formation of extracellular matrix (Avila 2006). 2) The phosphoinositide 3-kinases (PI3K)/Akt/mammalian target of rapamycin (mTOR) signalling pathway plays an important role in HCC tumour growth and survival and it is activated in 30–50% of HCCs. mTOR pathway can be dysregulated via abnormal activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), insulin-like growth factor 1 (IGF1R), mesenchymal–epithelial transition factor (MET), vascular endothelial growth factor receptor (VEGFR) or platelet-derived growth factor receptor (PDGFR). 3) The ERK/MAPK pathway regulates cellular proliferation, differentiation, angiogenesis and survival (Gollob 2006). This pathway can be activated through mutations in RAS oncogene or overexpression of growth factors, activated pathway contributes to HCC progression and metastasis (Whittaker 2010).

HCC is regarded as a heterogeneous disease and the cellular origin still remains uncertain. Animal experiments highlighted four groups of cells in the hepatic lineage which may respond to different carcinogenic regimens: (1) the mature hepatocyte in diethylnitrosamine (DEN) hepatocarcinogenesis model (2) The bile duct progenitor cells when furan model was used. (3) The ductular 'bipolar' progenitor cell in N-2-acetylaminofluorene (N-2-AAF) based regimens and (4) the periductular stem cell, which is the cell of origin of HCC in choline deficiency models (Sell 2002).

HCC is an aggressive malignancy and the mode of metastasis includes local invasion, lymphatic and haematogenous spread. The common sites of distant spread are lungs, adrenals and bone (Tamura 2001). Unfortunately when HCC presents with clinical symptoms, the tumour is usually advanced. The available effective treatments are only applicable to a relatively small proportion of patients diagnosed at an early stage except with surveillance.

1.2. Diagnosis: The diagnosis is based on a combination of clinical, laboratory, radiological and pathology examinations. Diagnostic confirmation and tumour staging is essential for the management of HCC. Unfortunately it is a silent disease with non-specific clinical symptoms precluding an early diagnosis. The delayed symptoms are mainly due to hepatic decompensation.

Imaging plays an important role in the diagnosis of HCC. Recent advances in radiology allows better characterisation of hepatic lesions, however accurate identification of sub-centimetre lesions in a cirrhotic background continues to be a challenge.

Serum α-fetoprotein (AFP): A diagnosis of HCC based on serum AFP alone is challenging as up to 20% of HCC do not secrete AFP irrespective of the size of tumour. In AFP producing tumours, the levels usually correlate with tumour size and volume at the time of diagnosis. Serum AFP level greater than 200ng/ml is highly specific for HCC in high-risk patients with suspicious lesions on imaging.

Ultrasound (USS): CT and MRI imaging has largely replaced USS due to its low sensitivity and positive predictive value. In current practise the role of USS is largely limited to a screening tool.

Multi-phasic Computerised tomography (CT): CT evaluation of suspected HCC is done by the use of 4-phase scanning that captures images before injection of contrast, during arterial, venous and delayed phases of intravenous contrast. Vascular supply to the lesion is the key pathologic factor in the differential diagnosis. The presence of arterial enhancement followed by delayed hypo-intensity of the tumour in the portal venous and delayed phases of CT scan is pathognomic of HCC.

Magnetic resonance imaging (MRI): Triple-phase dynamic contrast enhanced MRI uses similar concepts applied to CT in the diagnosis of HCC. The sensitivity and specificity are similar to multi-phasic CT scan.

Angiography: Only used selectively to define vascular anatomy prior to surgical resection or transarterial chemoembolization.

Positron emission tomography combined with computed tomography (PET-CT): PET-CT has limited application in the diagnosis of primary HCC. High levels of glucose-6-phosphatase in the liver cells dephosphorylates the tracer ^{18}flurodeoxyglucose (^{18}F-FDG) resulting in only 30-50% uptake. The use of dual PET-CT protocol using ^{11} C-acetate in combination with ^{18}F-FDG has improved the sensitivity in detecting HCC metastases but its role in primary HCC is still limited (Ho 2007).

Diagnostic laparoscopy with contrast enhanced intraoperative ultrasonography is reported to have greater sensitivity for detection of both liver lesions and extrahepatic metastases than conventional imaging but its role in routine practice has not gained support (Montorsi 2007). A chest CT and bone scintigraphy (optional) are performed to exclude metastatic disease prior to surgery, but these tests lack sensitivity for detection of small lesions and fail to identify micrometastases. A routine pre-operative biopsy is not performed in HCC due to the perceived risk of needle tract seeding of HCC cells (Durand 2001). This precludes the identification of potentially important prognostic variables, particularly microscopic vascular invasion and tumour differentiation that can be used to develop better risk stratification during pre-operative staging.

Screening for HCC: Unless diagnosed early HCC carries a dismal prognosis. The median survival of patients presenting with advanced HCC is less than 6 months (Llovet 2005).

Routine surveillance of high-risk cirrhotic patients increased the diagnostic rate of HCC, identified tumours at an early stage and reduced mortality (Zhang 2004, Chen 2003). A six monthly serum AFP combined with USS examination is recommended in screening high-risk patients for HCC (Thompson Coon 2007).

Tumour staging: Cancer staging is crucial to select an optimal treatment for individual patients, estimate the prognosis and evaluate the results. Patients with HCC constitute a special group where the applicability and efficacy of the treatments is also determined by the underlying liver cirrhosis. The conventional tumour node metastases (TNM) system used for oncological staging is therefore not applicable for HCC and may result in inaccurate prediction of survival.

The prognostic models for HCC should include: assessment of tumour burden, impairment of liver function, clinical performance status and choice of treatment (Briux 2005). Pre-operative tumour extent can be evaluated using contrast-enhanced MRI or helical CT of the abdomen, chest CT and an optional bone scan in advanced disease. Liver function is assessed using Child-Pugh scoring system and Model for End-stage Liver Disease score (MELD) and portal hypertension is graded by measuring hepatic-venous pressure gradient.

Several staging systems were developed incorporating some or all of the above mentioned parameters, but there is no worldwide consensus on a particular system (Verslype 2009).

The advantages and limitations in clinical application of these systems are discussed:

1) Okuda stage (Okuda 1985): It was the first staging system incorporating both tumour and liver function parameters and has been widely used in the last decade. The drawback was use of a broad classification for assessment of tumour burden (more or less than 50% of liver involvement). In the current era of early tumour diagnosis this classification is not adequate to stratify patients prior to treatment or subgroup according to tumour size.

2) French classification (Chevret 1999): This classification was developed using a prospective study including 761 patients. Five tumour and liver function variables were identified to stratify patients into 3 risk groups A, B and C. The major limitation was poor patient selection reflected by worse survival outcomes for best stages (Cillo 2004). In comparison with other staging systems it has limited prognostic capacity for early tumours.
3) Cancer of the Liver Italian Program (CLIP) score (Farinati 2000): This system was developed using a retrospective study. The score is calculated by adding four biochemical and tumour parameters and patients are divided into seven prognostic stages. It has been compared with Okuda and TNM stages and has better discriminatory power. The limitations were, like Okuda system, it cannot distinguish between apparently disparate tumour types and the survival figures from the external validation studies were variable.
4) The Chinese University Prognostic Index (CUPI) score (Leung 2002): This was developed in Hong Kong based on a study analysing the outcomes of 926 patients, the majority of whom were HBV positive. The issues were similar to that of the French classification, where the original and validation studies reported poor survival in patients with early stage disease.

5) The Japan Integrated Staging (JIS) (Liver cancer study group of Japan 2000): Includes both TNM and Child-Pugh classifications but lacks validation in Western countries.
6) The Barcelona- Clinic Liver Cancer (BCLC) staging (Llovet 1999): This was developed using variables related to tumour stage, liver function status, physical status and cancer related symptoms by the Barcelona group. It is the only system that incorporates all the EASL variables and considers type of treatment as prognostic variable (Bruix 2001). Many prognostic models (CLIP, French, Okuda, CUPI) only included advanced or small groups of radically treated cases leaving behind the cohort of patients with early disease amenable for radical treatments. BCLC addressed the issue by dividing the cases into early, intermediate, advanced and end-stage linked with a treatment algorithm. BCLC has been validated to be the best prognostic model when applied to patients treated with potentially radical treatments (Cillo 2004).

The heterogeneous methodology and populations included has resulted in staging systems with no universal acceptance. Three of the systems (BCLC, CLIP, and JIS) have been validated in different patient cohorts. The Cancer of the Liver Italian Program (CLIP) score has shown to be of particular value in advanced cases in a French population (Collette S, 2008). The pTNM system is based on the pathology report and may be relevant to stratify patients for studies of adjuvant treatments. Although there is no single ideal staging system for HCC, the Barcelona-Clinic Liver Cancer (BCLC) group 5 stage system has been adopted by many centres as it is combines the most practical evaluation of liver function with tumour staging to provide an algorithm of treatment. The system identifies those patients with early HCC who may benefit form curative therapies (stage 0 and A) and those at intermediate (stage B or C) or advanced stage who may benefit from palliative treatments, and finally those with a very poor life expectancy (stage D).

1.3 Management of HCC: Assessment of both the tumour and the underlying liver is undertaken by a multidisciplinary team. The aim of treatment is to remove the neoplasm while preserving liver function. The factors dictating the therapeutic approach are severity of underlying cirrhosis, portal hypertension and the overall tumour burden. Available options include surgical resection, orthotopic liver transplantation (OLT), loco-regional therapies, chemotherapy and biological therapies.

Surgical resection: Surgical treatment is offered to patients with no underlying cirrhosis or those with Child class A or B liver functional status. Adequate estimation of pre-operative liver function and planning the extent of hepatectomy improves outcomes of surgical resection. Portal hypertension is an independent predictor of survival and if significantly elevated is considered a contraindication for hepatectomy (Bruix 2005). Hepatectomy is the treatment of choice in patients who are not suitable candidates for transplantation due to large tumour size, macrovascular invasion, advanced age or associated co-morbidities (Poon 2004). Although it offers good long term outcomes in patients with good hepatic reserve, this group of patients accounts for less than 5% of HCC in the Western population.

Advanced surgical and anaesthetic techniques have significantly reduced the post-operative mortality and morbidity, although disease recurrence is a major concern. Cirrhosis is known to increase the risk of recurrence after resection of HCC and the 5-year recurrence rate exceeds

50% in patients with underlying cirrhosis (Poon 2000). It has been shown that intra-hepatic tumour recurrence is the most common form (60% to 70%), although systemic metastases occur in the lungs (37%), pleura (26%) and bone (11%) (Shirabe 1996).

Many authors have attempted to correlate the patterns of intrahepatic metastasis with the time interval between recurrence and liver resection. It has been demonstrated that early recurrence within the first 2 years after resection of HCC is associated with tumour related factors such as high tumour grade, microvascular invasion and microsatellite lesions, whereas late recurrence is more likely to represent multicentric hepatocarcinogenesis in a cirrhotic liver (Portolani 2006).

A variety of factors have been shown to have prognostic significance by predicting tumour recurrence. High alpha-fetoprotein level (>400ng/ml), poorly differentiated tumour and microvascular invasion on histo-pathological examination are associated with early recurrence. Male gender, older age, high transaminase levels, multiple primary tumours, and high alpha-fetoprotein level have all been linked with late recurrence (Imamura 2003, Poon 2000). Of all these identified risk factors from retrospective studies only tumour size and vascular invasion have been shown consistently to influence prognosis (Poon 2000; Tsai 2000). A preoperative histological or molecular examination of tumour and adjacent non-tumour tissue is needed in combination with radiological staging to accurately predict tumour recurrence (Villaneuva 2011). At present the associated risk of needle track recurrence with a preoperative biopsy precludes its routine use in clinical practice (Llovet 2001). Early detection and effective treatment of HCC recurrence can prolong patient survival. Aggressive management of intrahepatic recurrence with re-resection or loco regional therapies such as transarterial chemoembolization (TACE), radio-frequency ablation (RFA) and percutaneous ethanol injection (PEI) or salvage liver transplantation may improve outcomes after surgical resection (Poon 2007). Unfortunately the majority of patients with intrahepatic recurrence are not candidates for re-resection because of multiple recurrences or inadequate liver functional reserve. Data to support a role for transarterial chemo-embolization either preoperatively or postoperatively, to prevent or treat recurrent HCC is unconvincing (Chua 2009 Schwartz 2005; Tanaka 1999). Currently there is no established effective adjuvant therapy in preventing recurrence after resection of HCC. Approaches such as transarterial iodine-131, interferon and adoptive immunotherapy require further evaluation by more randomized trials (Sun 2003). Although the risk factor analysis provides a rough guide to assess the risk of recurrence, it cannot specifically predict in an individual patient. The biological behaviour of HCC remains largely unpredictable and the risk of recurrence due to an occult microscopic disease persists, an understanding of micrometastases to develop possible targeted treatments is essential to prevent early tumour recurrence.

Liver transplantation (LT): LT has changed the treatment strategy for patients with small volume disease and no extra-hepatic involvement. It treats both the tumour and underlying cirrhosis providing the best therapeutic option with a reasonable long-term survival benefit (Mazzaferro 1996). The early experience with OLT in the late 1980s was associated with a high early recurrence rate and 5-year survival rates ranging between 18%-40%. Mazzaferro et al, established the Milan selection criteria for LT, which identified a subgroup of patients with radiologic evidence of a single tumour ≤5 cm in diameter, or two to three tumours ≤3cm in diameter had 5-year recurrence-free survival rates of 75 and 83%, respectively. United Network for Organ Sharing (UNOS) staging system for allocating organs for OLT in the United States adopted the Milan criteria to allocate organs for LT. More recently it has been considered that the Milan criteria may be too restrictive of access to LT and the extended University of California, San Francisco criteria (UCSF) i.e. single lesion less than 6.5 cm or up to three lesions with the largest lesion less than 4.5 cm with a total tumour diameter less than 8 cm has been introduced (Yao 2004). Although OLT offers the theoretical advantage of complete removal of the tumour as well as the underlying disease, tumour recurrence is a limitation even in patients meeting Milan criteria (Schlitt 1999). Post-transplant tumour recurrence is reported as 15%-25% and is a significant cause of death following LT. The most common sites of tumour recurrence are liver followed by lung and bone (Zimmerman 2008).

Predicting tumour recurrence using clinco-pathological variables can help in better allocation of the limited supply of donor livers and also initiate adjuvant treatments for the at-risk groups. Several studies attempted to develop scoring systems aimed at predicting post-transplant recurrence. Size of the tumour and micro-vascular invasion noted in the explant specimen were found to be the strong predictors of recurrence. In the absence of extra-hepatic disease, micro-vascular invasion is the most powerful predictor of HCC recurrence (Ramos 2005, Pawlik 2006, Parfitt 2007). Poor tumour differentiation is associated with microscopic vascular invasion (Esnaola 2002, Zavaglia 2005). Size of the tumour ≥ 5cm is an independent predictor of recurrence and a surrogate marker for vascular invasion (Ramos 2005, Pawlik 2006, Parfitt 2007). There is increasing evidence indicating that maximum diameter of the largest nodule is superior to the number of nodules in predicting post-OLT tumour recurrence. However there is discrepancy in defining a cutoff value for the maximum diameter among these studies. Marelli et al. and Zimmerman et al. used a cutoff of 35 mm for the main nodule size while Esnaola et al. showed that tumour size > 4 cm in the explant liver was an independent predictor of micro-vascular invasion. Shetty et al. found that explant tumour diameter >3 cm was significantly associated with recurrence. The Metro ticket data, presented at the International Liver Cancer Association (Barcelona 2007), also suggested that maximum diameter of tumour nodule(s) is more predictive of tumour recurrence than the number of nodules. Unfortunately, the tumour characteristics needed to accurately predict recurrence can only be assessed conclusively at explant histopathological examination. A pre OLT biopsy may not effectively demonstrate micro-vascular invasion or assess tumour grade accurately because of the known histological heterogeneity in large tumours (A 2001, Pawlik 2007). In addition percutaneous biopsy is associated with a risk of tumour seeding (Stigliano 2007).

In patients who meet the strict selection criteria, post-operative tumour recurrence must reflect the presence of undetected microscopic extra hepatic disease (micrometastases) at the time of transplantation. Detecting micrometastases preoperatively may help to identify a subgroup of patients who are at a higher risk of tumour recurrence (Sutcliffe 2005). These disseminated cells often show different properties to those of the primary tumour. Isolating, characterising and understanding the behaviour of these disseminated cells may potentially lead to the development of targeted therapies for these high risk patients.

Loco-regional therapies:

Transarterial chemoembolization (TACE): TACE offers palliative benefits for patients with large volume disease (Chok 2006). Chemo-embolization delivers chemotherapy directly to the tumour via the arterial supply thus avoiding systemic toxicity. Doxorubicin, mitomycin and cisplatin are the most commonly used chemotherapy agents. Embolizing agents usually gelatin or microspheres that are administered along with chemotherapy cause ischaemic injury to tumour cells rendering them more sensitive to cytotoxic agents. TACE causes a significant delay in tumour progression and vascular invasion, a partial response is achieved in 15-55% of patients (Llovet 2002). Other available modalities of embolization includes arterial chemoembolization with degradable starch microspheres and continuous arterial Infusion of 5-Fluorouracil, selective internal radiation therapy using yttrium-90 microspheres (SIRT).

Evidence from randomized controlled trials showed high rates of tumour response and a survival benefit with TACE and RFA when applied as primary treatment (Cho 2009, Cabibbo 2010). Use of such modalities in patients awaiting LT has been considered to improve post-LT survival, down-stage advanced HCC to within Milan criteria and prevents waiting list drop-out (Frangakis 2010, Schwartz 2007).

Although there are no randomised controlled trials to support the role of pre-transplant treatments, most centres have accepted the available indirect evidence as a basis for their routine application.

Ablative modalities: Several percutaneous ablative treatments were developed for patients who are not suitable for surgical resection or transplantation. Percutaneous ethanol injection (PEI) & radiofrequency ablation (RFA) are the most common modalities used in clinical practice. Other alternative treatments include percutaneous acetic acid injection (PAI), microwave coagulation therapy (MCT), laser interstitial thermal ablation therapy and cryoablation therapy. The response to ablative therapy is dependent on baseline liver function and the size of the tumour. In patients with Child-Pugh grade A, a response rate of 80% can be achieved with tumours less than 3 cm in diameter. PEI and RFA can provide 5-yr survival rates of 40–70% in well selected patients (Lencioni 2005, Sala 2004, Omata 2004).

PEI technique involves slow injection of absolute or 95% alcohol into the lesion thus causing tumour necrosis. It is safe and highly effective for tumours less than 3cm in size. Radiofrequency ablation causes tumour necrosis by placement of a probe that generates heat by high frequency ultrasound directly into the lesion (Morris 2000; Ohmoto 2000). It can be used to effectively ablate a tissue of as much as 3 cm or more in diameter. RFA can be applied percutaneously, laparoscopically or during laparotomy. RFA is reported to achieve slightly better response rates with a significantly fewer number of sessions when compared to PEI. A five-year survival of 40-70% has been reported and the response to ablative therapy depends on the tumour size and Child-Pugh grade (Germani 2010).

Molecular therapies: Molecular therapies targeting signalling pathways are currently under investigation. These agents either block the pathways related to cell proliferation and survival or signals related to dissemination of the disease. Sorafenib, an oral multikinase inhibitor targets cell proliferation and angiogenesis, both pathways that are the involved in hepatocarcinogenesis.

Sorafenib, an oral multikinase inhibitor targets cell proliferation and angiogenesis, both pathways that are the involved in hepatocarcinogenesis. SHARP study demonstrated that Sorafenib improved median survival from 7.9 months to 10.7 months and slowed time to progression from 2.8 to 5.5 months (Llovet 2008). Based on these results, it is regarded as the standard therapy for metastatic disease and for HCC progressing despite optimal locoregional therapy. A number of ongoing studies are establishing Sorafenib's adjuvant role in resection, local ablation and TACE. Early results from a recent study showed that concurrent Sorafenib and TACE treatment resulted in an overall median survival rate of 18.5 months (16.1-20.9 months) with acceptable side effect profile (Cabrera 2011). The results from studies targeting Ras/MAPK, Akt/mTOR pathways as well as monoclonal antibodies against vascular endothelial growth factors (VEGF) are pending (Llovet et al 2008).

2. Micrometastases/ Disseminated tumour cells (DTC):

Intra-hepatic tumour recurrence following curative surgical procedures suggests that microscopic tumour metastases are present prior to surgery. Microscopic disseminated tumour cells that have the potential to develop into clinical metastases have been labelled as ‘micrometastases’ (Sloane et al, 1980). Although a formal definition does not exist, micrometastasis is defined as microscopic deposits of malignant cells (smaller than 2mm) that are segregated from primary tumour and depend on neovascular formation for propagation. They are thought to originate from single tumour cells and are readily missed by routine pre-operative staging investigations. The fundamental biological characteristic which distinguishes them from macroscopic metastases is the absence of a specific blood supply. Micrometastatic cells depend on passive diffusion for oxygen and nutrition and this limits their growth to 2-3mm in size. They may remain in a dormant state for prolonged periods with no net growth (Pantel 1999). Disseminated cells may also persist in a viable state in the peripheral circulation and/or distant organs, such as lung and bone. They are detectable by very sensitive methods even in the absence of clinical metastases demonstrated by conventional staging procedures (Schlimok 2000).

Micrometastases can be isolated in patients with epithelial malignancies and has prognostic significance. There is growing evidence of DTC in the peripheral blood, lymph nodes and bone marrow (BM) in a variety of solid organ cancers including breast, colorectal, oesophageal and pancreatic cancers. Knowledge regarding the presence of DTC in patients with HCC is limited. Several techniques have been developed to identify these cells and the two common methods are immunocytochemical staining and/or PCR analysis.

2.1 Micrometastases and Hepatocellular Carcinoma

Recurrence of HCC following potentially curative treatments in carefully selected patients indicates that DTC are dispersed from the primary tumour prior to surgery and persist in a viable state in the liver, peripheral circulation and/or distant organs. DTC in patients with HCC appear to have a predilection for the allograft or the liver remnant following surgical resection which remains the most common site (80%) of tumour recurrence (Otto 1998, Philosophe 1998, Weimann 1999, Yao 2001, De Carlis 2001, Klintmalm 1998, Schlitt 1999, Tamura 2001). There is considerably less evidence in support of a role for micrometastases in HCC recurrence when compared to other epithelial malignancies.

The phenomenon of micrometastases is well studied in breast cancer. It has been shown that the presence of disseminated cells in bone marrow or peripheral blood is associated with a poor prognosis (Riethdorf 2008). Detection of micrometastases using laboratory-based techniques has some potential advantages. First, they may be isolated from samples of bone marrow or peripheral blood, which can be obtained safely for assessment of tumour staging. Second, micrometastases status may be evaluated preoperatively and at intervals postoperatively to assess the therapeutic response in patients receiving adjuvant treatment.

In HCC, several studies have focused on the use of reverse transcriptase polymerase chain reaction (RT-PCR), to detect either albumin mRNA or Alpha feto-protein (AFP) mRNA in blood and bone marrow samples (Kienle 2000, Aselmann 2001, Ijichi 2002). These have been used as markers of circulating disseminated HCC cells (Jeng 2004, Marubashi 2007). RT-PCR is highly sensitive for detecting albumin and can identify one disseminated HCC cell in a sample of 10 million peripheral blood cells, however, is associated with a high false positive rate (Kubota 2002). RT-PCR for AFP is of less value as a marker for micrometastases in patients with HCC as it is non-specific and can be detected even in patients without HCC as well as AFP-negative HCC (Sutcliffe 2005). Immunocytochemical detection assays use monoclonal antibodies that bind specifically to tumour associated proteins expressed by tumour cells. These assays are capable of detecting a single metastatic cell amongst millions of normal cells. Sutcliffe et al demonstrated that immunocytochemistry (ICC) using a monoclonal antibody against hepatocyte specific antigen (Hep Par-1) is a promising technique for detection of HCC micrometastases in the bone marrow. It was also shown that ICC is sensitive and superior to RT-PCR in terms of specificity and clinical relevance. Although these studies demonstrated techniques to detect DTC the clinical significance of their occurrence is still unclear. The biological properties of disseminated cells are poorly understood and the molecular mechanisms that regulate their cell growth, survival, and immune evasion are unknown. There is indirect and conflicting evidence suggesting that these cells may be cleared by the host immune system, may remain dormant for prolonged periods before developing into clinical metastases, or may not have potential to develop into clinical metastases (O'Sullivan 1997, Braun 1999). Studies involving breast, colorectal and oesophageal cancers reported that tumour recurrence is not seen in every patient with detectable micrometastases. In our previous study cohort of 18 HCC patients undergoing transplantation, 7 out of 12 patients with Hep Par-1 positive micrometastases were alive and disease free at a median follow-up of 37 months (Range 1-81 months) (Sutcliffe 2005).

To evaluate the clinical relevance and assess the malignant potential of DTC it is essential to isolate and study them using an *in vitro* culture model. Pantel et al. (1995) described a novel approach to isolate and immortalize micrometastatic cells in breast cancer, however, there is very little available literature on *in vitro* culture techniques using non-immortalised cells. Preoperative detection of micrometastases in patients with HCC and the study of the mechanisms that favour tumour recurrence are key areas for research that may have clinical and therapeutic implications.

3. Stem cells and carcinogenesis:

Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and generate mature cells of a particular tissue through differentiation. The cellular origin of tumours remains an important question. Recent evidence suggests that stem cells are implicated in tumour development as well as metastases (Reya 2001). They possess unique cellular mechanisms to account for the therapeutic resistance and recurrence of the tumours. Stem cells are the only cells that have the ability to persist in the tissue for a sufficient length of time to acquire the necessary genetic changes for neoplastic transformation and have enough plasticity to undergo changes required for dissemination at distant sites.

Stem cells are generally defined by an ability to differentiate into multiple cell lineages and self-renewal and contribute to not only organogenesis but also to regeneration in response to tissue and organ injury. Recent advances in stem cell biology have allowed the identification and characterization of stem cells in a variety of tissues and organs. In the setting of chronic inflammation, progenitor or stem cells within the peripheral tissue are forced to undergo multiple rounds of cell division causing DNA damage, accumulation of mutations and the emergence of malignant cells. (ZHAO and New hope for cancer) The stem cell origin of cancer was suggested by the pathologists Recamier and Virchow over 100 years ago and was based on the finding of histological similarities between a developing embryo and teratomas (Sell 2004). Teratocarcinomas are derived from embryonic germ cells which can differentiate into almost all cell lineages. These cancer cells can divide rapidly and are immortal, the properties which are similar to embryonic stem cells.

The isolation of stem cells from epithelial tumours, such as breast (Al-Hajj 2003), prostate (Collins 2005), ovary (Szotek 2006), pancreas (Li 2007) and colon (Ricci-Vitiani 2007), have supported their involvement in carcinogenesis.

Currently there are two models for the cellular origin of cancer. The stochastic model indicates that every cell in a tumour cell population has the ability to act as a cancer stem cell, but only those with a capacity for self-renewal would have the ability to sustain neoplastic growth. The alternative hypothesis, namely, the hierarchical model describes that only a biologically distinct subset of the total malignant cell population called the cancer stem cells (CSC) are capable of initiating a tumour. Although both models share a common idea that only a limited number of cells within a tumour will initiate cancer, their biological principles and ultimately the clinical implications are very different. According to the stochastic theory, the cells within a tumour are relatively homogeneous and cytogenetic changes that lead to neoplasia are operative in all cells. Thus, research should be focussed on the entire tumour cell population to understand carcinogenesis pathways and develop new therapies. The stem cell model, however, assumes that the tumour cell population is heterogeneous and the sub-group of tumour-initiating cells are biologically and functionally distinct. The clinical implication from this model is that the elimination of all CSCs will terminate the growth of the tumour, and that failure to do so could result in tumour recurrence.

Recent studies of human breast and neurological malignancies have isolated a minor phenotypically distinct tumour cell population that have self-renewal capacity and are able to generate tumour (Al Hajj 2003, Singh 2004). There is growing evidence supporting the origin of cancer as a hierarchical disease whose growth is sustained by a biologically distinct group of CSCs (Wang 2005).

3.1 Stem cells in liver regeneration and carcinogenesis

Human hepatocytes have a life span of over a year and seldom proliferate under normal physiological conditions. Alterations to liver mass brought on by surgical resection or by viral or chemical injury cause the quiescent hepatocytes to proliferative and restore functional liver substance. The degree of inflammation and cell loss dictates the type of cells involved in replenishing the cell mass. Replication of hepatocytes is sufficient to restore the liver mass following a surgical hepatectomy, however severe degrees of inflammation can result in expansion of bipotential progenitor cells which can differentiate into hepatocytes and biliary cells. These cells have been described as “intermediate hepatobiliary cells” and “hepatic progenitor cells” or the human equivalent of rodent “oval cells” originating from the terminal branches of the intralobular biliary tree- the canals of Hering. The degree of stem cell and intermediate hepatocyte activation correlates with the degree of inflammation and fibrosis (Lowes 1999, Roskams 2003).This progenitor cell compartment is a potential target for carcinogenesis in the presence of chronic liver inflammation and growth factors.

There has been a growing interest in the role of stem cells in liver cancer (Alison 2005, Libbrecht 2006, Sell 2008). Current evidence from animal models of hepatocarcinogenesis suggests that only a minority of HCC are derived from mature hepatocytes. The Solt–Farber model demonstrated that ductular ‘bipolar’ progenitor cells give rise to HCC in an N-2-acetylaminofluorene (N-2-AAF) based regimen while the choline deficiency model proposed that the peri-ductular oval stem cell gives rise to HCC. The evidence from chemical hepatocarcinogenesis animal models, the presence of bipotential hepatocyte and cholangiocyte cells in 28% to 50% of HCC tumour specimens, the relative resistance to chemo-radiotherapy and tumour transplantability suggests a role for stem cells in the origin and metastases of human HCC.

3.2 Role of bone marrow in liver regeneration and hepatocarcinogenesis:

There is growing evidence to support the presence of an extra hepatic source of progenitor cells which participate in liver generation. It is hypothesised that the type and degree of liver injury determines the recruitment and mobilisation of such cells, however, the exact underlying pathways are still unclear. Evidence from studies demonstrating the expression of haematopoietic stem cell markers such as Thy1, CD34 and c-kit by hepatic progenitor cells (HPC) (Petersen 1998 Omori 1997 Baumann 1999) and differentiation of haematopoietic stem cells into hepatic progenitor cells (HPC) in animal models (Peterson 1999) suggests a possible bone marrow origin of HPC.

Over recent years bone marrow (BM) stem cells have generated interest with the discovery of two properties namely transdifferentiation; the capacity to generate different types of tissue cells and differentiation plasticity, the ability to choose multiple differentiation pathways. Korbling et al. showed that stem cells from the peripheral blood of patients who underwent allogenic peripheral-blood stem cell transplant could differentiate into cells of liver, gastrointestinal tract and skin. Several *in vivo* and *in vitro* models have demonstrated the ability of bone marrow stem cells to differentiate into hepatocytes (Theise ND 2000, Schwartz RE 2002 Lagasse E 2002). There are conflicting views on whether the hepatocytes are a result of cell fusion or transdifferentiation of BM stem cells.

The role of bone marrow stromal cells in normal hepatic maintenance or the pathways that could result in their mobilisation in response to chronic injury or malignancy in humans has not been addressed clearly to date. The most successful model in terms of the re-population and functionality of bone marrow-derived hepatocytes is the FAH (-/-) mouse, an animal model of tyrosinemia type I in which intravenous injection of BM cells rescued the mouse restoring its liver function (Lagasse 2000). A similar phenomenon was noted in sex mismatch liver transplant recipients where BM derived hepatocytes were identified in liver biopsy specimens in patients with severe liver injury (recurrent Hepatitis C) (Newsome 2003).

It was hypothesised that persistent liver injury initiates recruitment of mesenchymal/haemopoetic stem cells from the bone marrow (Lagasse 2000, Korbling 2002). Inflammatory mediators, growth factors and SDF-1 /CXCR4 signalling pathways are implicated in the homing of these cells to the liver to aid in liver regeneration (Kallis 2007). Furthermore, there is evidence implicating BM derived cells in the liver fibrogenic response. Sex mismatch bone marrow transplant mice models exposed to chronic carbon tetrachloride (CCl4) poisoning and a model of cholestatic liver disease in bile duct ligated mouse demonstrated that myofibroblasts associated with septal scars were BM derived. Paradoxically some studies showed therapeutic benefit of BM derived cells in ameliorating liver fibrosis in animal models (Sakaida 2004). Gaia et al. demonstrated that bone marrow derived stem cells can be mobilised in patients with end stage liver disease with the administration of granulocyte colony-stimulating factor (G-CSF) and this correlated with an improvement in clinical their condition.

It is now widely accepted that cancer is a disease of stem cells and several experimental models were developed to study the role of stem cells in epithelial malignancies. The source of these cancer stem cells (CSCs) is thought to be either peripheral stem cells located in the neoplastic organ or BM derived cells (BMDC). BMDC are thought to act as a second line of defence in circumstances where the peripheral stem cells are subjected to atrophy and cell loss as a result of ongoing chronic injury and inflammation. BMDC share similar cell surface markers and follow the same chemotaxis and metastatic pathways used by CSCs, they can engraft into the stem cell niche and assume tissue stem cell function in the event of their atrophy. BMDC are inherently mutagenic and the presence of on-going inflammation and injury transforms them into cancer stem cells.

The involvement of BM cells in carcinogenesis was demonstrated beyond just theory by Houghton et al (2005). In their gastric carcinoma H. felis/C57BL/6 mouse model it was shown that chronic infection of mice with Helicobacter, a known carcinogen, resulted in repopulation of the stomach with BM derived cells. These cells subsequently progressed through metaplasia and dysplasia to intraepithelial cancer. Avital et al (2003) highlighted the contribution of bone marrow stem cells in the development of epithelial malignancies in 4 sex mismatch bone marrow transplant recipients.

Whether HCC derives exclusively from epithelial cells within the liver, or has a bone marrow derived stem cell contribution, is unanswered. Furthermore, whether such cells affect tumour phenotype (e.g. metastatic potential or treatment responsiveness) is unclear.

4 Clinical applications of isolating disseminated tumour cells (DTC):

The best evidence from breast cancer research suggests that DTC are associated with poor outcomes, however, information regarding their biological and molecular properties is very limited. The DTC population is very heterogeneous and only a small subset of these cells might develop into overt clinical metastases. The expression of stem cell phenotypes and their association with cancer stem cells in breast cancer is still unclear.

To increase the diagnostic accuracy or monitor the effectiveness of systemic adjuvant treatments more studies are needed at a cellular level (Pantel 2009). Isolation and *in vitro* culture of DTC will provide an opportunity to characterize their biological properties and molecular profile and thus may further lead to development of targeted therapies. (Pantel 1995, O'Sullivan 1999).

An in depth knowledge on DTC in patients with HCC will help improve our current staging systems, may help predict survival, assess the therapeutic response to down staging and the use of adjuvant treatments. It will also assist the development of targeted therapies to reduce the likelihood of tumour recurrence.

Hypothesis:

The aim of treatment in HCC is to prolong survival and improve the quality of life by eradicating the tumour while preserving liver function. Surgical resection or transplantation are potentially curative treatments in well selected patients, however, the risk of tumour recurrence still remains a major limitation. It is essential that these candidates are more accurately stratified according to risk of postoperative recurrence for effective use of available treatments. The current predictors of recurrence are mainly based on histological and molecular examination of the tumour tissue but this information is not routinely available prior to radical surgical treatments. Intrahepatic tumour recurrence especially following LT that offers treatment for both the tumour and underlying cirrhosis raises the possibility of microscopic disseminated disease. The presence of micrometastases has been associated with disease recurrence but their biological behaviour remains speculative. To-date there is no effective adjuvant treatment to reduce the risk of tumour recurrence and the poor response to systemic chemotherapy may be explained by the low proliferative state or the presence of effective cell repair mechanisms. An understanding of these micrometastatic cells is essential to develop targeted biological therapies that may offer more effective adjuvant treatment in these patients.

AIMS:

a) Establish a protocol for *In vitro* culture of disseminated hepatocellular carcinoma cells from peripheral blood

b) Understand their biology as well as establish their origin by immunocytochemistry and molecular techniques.

c) Assess the malignant potential of disseminated hepatocellular carcinoma cells using a suitable animal model.

CHAPTER 2: MATERIALS AND METHODS

2.1 Alphabetical List of Reagents

See appendix

2.2 Ethical Approval:

Ethical approval for this study was granted by King's College Hospital Research Ethics Committee on 12th June 2001. An amendment to permit collection of additional samples from patients without hepatocellular carcinoma was approved on 1st February 2002.

2.3 Patient Recruitment:

Potential subjects were identified from the King's College Hospital Liver Transplant Waiting List, wards and outpatient clinics. Detailed information on the purpose of the study and the associated risks with sample collection were provided to all who were invited to take part in our study. Those willing to participate in the study after reading the information and asking relevant questions were recruited. A written consent was obtained in all cases in accordance with the local ethical committee regulations. The subjects were permitted to withdraw from the study at any stage with no bearing on their clinical treatment.

For the purpose of the study the recruited cohort was divided into 3 groups; Group 1- Patients with a clinical diagnosis of HCC, Group 2- Patients with end stage liver disease but with no evidence of HCC and Group 3- healthy human volunteers. The cases from group 2 and 3 acted as controls.

2.4 Data Collection

Demographic and essential data including preoperative investigations, radiological staging, adjuvant treatment modalities and postoperative histology were collected and stored on a password protected excel database. The details are presented in Table 1.

Table 1: Demographic and clinical parameters:

Clinical variables	Pre-operative interventions	Tumour characteristics (Radiology and post-operative histopathogy where available)
Age Sex Underlying liver disease Hepatitis status Serum AFP levels at presentation Significant past medical history MELD score	Liver biopsy Trans-arterial chemoembolization Radiofrequency ablation	Size of the tumour Number of nodules Vascular invasion(Micro/Macro) Degree of differentiation

2.5 Acquisition and processing of peripheral blood samples:

Peripheral venous blood samples were obtained from the anterior cubital fossa of patients with HCC and control groups (20ml). The samples were collected under aseptic precautions in EDTA vacutainer bottles and were processed immediately.

2.5.1 Density centrifugation:

The peripheral blood was carefully layered onto Histopaque-1077 at a ratio of approximately 2 volumes of histopaque for each volume of blood. The mixture was centrifuged at 1200 RPM and 4°C for 20 minutes. The buffy coat with mononuclear cell fraction was aspirated and washed twice with PBS at 1500 RPM for 5 min each. The supernatant was discarded and the cell pellet was resuspended in the prepared culture medium. The cells were counted using a haematocytometer (Merck, UK) and the viability was checked with tryphan blue. 10^3 cells were cytospun on labelled polyprep glass slides (Sigma, UK) using cytocentrifugation (Hettich, Germany) at 3500 RPM for 6min. The pre-culture slides were air dried for 1hour and stored at -80°C for future analysis.

2.5.2 *In vitro* cell culture:

The cell suspension obtained from density centrifugation was resuspended in 10ml of Alpha MEM culture medium that was enriched with 20% fetal calf serum, 1% L-glutamine and 1% antibiotic and antifungal reagents. The mixture was transferred into a T75 culture flask and incubated at 37°C and 5% CO2. Culture medium was replaced after 12 hours. To minimise the loss of any disseminated cells the old medium was centrifuged at 1500 RPM for 5min and the resultant cell pellet was resuspended and added to the flask. Human recombinant hepatocyte growth factor (HGF) was added at 10ng/ml on day 1, 2 and weekly thereafter.

2.5.3 Cell detachment:

Cell detachment using trypsin EDTA: The old culture medium was removed and the flasks were washed twice with PBS. 3ml of freshly thawed ice-cold trypsin EDTA was added to a T75 flask and incubated at 37°C for 3 minutes. The reaction was terminated by addition of culture medium with fetal calf serum. The loosely adherent cells were detached from the surface with rigorous shaking. The procedure was repeated 3 times to recover the maximum number of cells.

Cell detachment using accutase: The old culture medium was removed and the flasks were washed twice with PBS. 10 ml of ice-cold accutase (Sigma, UK) was added to a T75 flask and incubated at 37°C for 5-10minutes. The procedure was repeated up to 4 times, it was noted that accutase could be used up to 45 minutes with no adverse effects on cell viability.

2.5.4 Storage of cultured cells:

The detached cells were centrifuged at 1500 RPM for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in culture medium to make a volume equivalent to 1ml. After assessing the cell count and viability a part of the cell suspension was used to make cytospin slides as described in 3.4. 1000 cells were included in each slide and the slides were air dried for 1 hour and stored at -80°C. The remaining cells were cryopreserved.

Cryopreservation: A freezing medium was made using one part dimethylsulphoxide in one part fetal calf serum and 2 parts culture medium. The cell suspension and ice-cold freezing medium were added to labelled sterile cryovial tubes at equal volumes to make a resultant volume of 1ml. The cryovials were stored in a cryopreservation canister containing 70% isopropanol at -80°C for 24-48 hours to allow the reduction of temperature by approximately 1°C per minute, subsequently the samples were stored in a liquid nitrogen tank.

2.6 Preparation of positive control cells:

Type of cells:

Primary human hepatoma cells

Human hepatoma cell line

Liver endothelial cells

Primary human hepatocytes

Bone marrow stem cells

Human colon cancer cell line [CaCo-2].

2.6.1 Culture of human hepatoma cells

Tumour tissue was obtained from fresh hepatectomy specimens, those who underwent pre-operative TACE or any ablative procedures were excluded. Macroscopically viable tumour was sliced with a sterile scalpel avoiding the central areas of the tumour. The tissue was collected in Alpha MEM medium and transferred to the laboratory over ice. The specimen was chopped into 1 mm^3 size pieces and washed twice at 1400 RPM for 10 minutes. The pieces were immersed in Type 4 collagenase (0.8 mg/ml) and incubated at 37°C for 2 hours with gentle agitation every 30 minutes. Following incubation partly digested pieces of the tissue were mashed with a 2ml syringe plunger; the resultant mixture was filtered through a 0.7µm sieve. The filtrate was centrifuged at 1400 RPM for 10 minutes and the cell pellet was washed twice. Three million cells with 40% viability were isolated from 10 g of tumour tissue, the cells were transferred into T75 flasks for further culture using the medium as described in 3.5.3. The flasks were incubated at 37 °C and 5% CO_2 for 5 days and the medium was renewed on alternate days. The cells were detached at confluence using trypsin EDTA. A proportion of cells were used for preparation of the cytology slides and the rest were cryopreserved.

2.6.2 Culture of human hepatoma cell lines:

Hep G2 cell lines were purchased commercially from the European collection of cell cultures (ECACC). The cryopreserved samples were quickly thawed at 37°C, after cell count and viability check, 10^6 cells were introduced into a T75 culture flask. The cell lines were maintained in the culture medium as described in Appendix VI. The flasks were incubated at 37°C in 5% CO_2 and culture medium was renewed every 2 days. At 80% confluence the cells were detached using 3 ml of trypsin EDTA and incubated at 37 °C for 3 minutes. The detached cells were washed twice with culture medium containing fetal calf serum. The cell pellet was resuspended in an appropriate amount of culture medium for cryopreservation and preparation of positive control slides for immunocytochemistry. Cytospin slides were prepared using the protocol described in 2.5.1.

2.6.3 Liver endothelial cell culture:

20 g of liver was obtained from donor organs that were deemed not suitable for transplantation due to the degree of steatosis. The liver tissue was sliced into $1mm^3$ size pieces and washed twice at 1400 RPM for 10 minutes. The pieces were then immersed in collagenase type 1a (0.8 mg/ml) and incubated at 37˚C for 1 hour with gentle agitation every 15 minutes. Following incubation the resultant digested mixture was filtered through a 0.7 µm sieve. The filtrate was centrifuged at 50g for 5 minutes to exclude hepatocytes. The supernatant was transferred into a 50ml falcon tube with Minimal Essential Medium (MEM) and centrifuged at 1400 RPM for 10 minutes, the cell pellet was resuspended in a freshly prepared culture medium favouring endothelial cell growth (Appendix VI). The cell mixture was transferred into collagen coated nine well plates and incubated at 37˚C in 5% CO2. The medium was changed every 2-3 days and the endothelial cell growth was monitored using cell morphology. At 80% confluence the cells were detached using 1ml of accutase per well and incubation at 37 ˚C for 10 minutes. The detached cells were washed with culture medium, the resultant cell pellet was resuspended in an appropriate amount of culture medium for sub-culture. The culture conditions used selected for endothelial cell growth in preference to other cell types (e.g. Fibroblasts, Kupffer cells, smooth muscle cells) yielding a purified culture of hepatic endothelium (>90%) after 2-3 passages. Cells from 3rd or 4th passage were used to prepare control slides for immunocytochemistry using our protocol described in section 2.8.2. Surplus cells were cryopreserved for future use.

2.6.4 Primary human hepatocytes:

The normal human hepatocytes were a donation from the hepatocyte transplantation group (Institute of Liver Studies, King's College Hospital). Hepatocytes were isolated from cadaveric donor livers that were deemed not suitable for transplantation due to steatosis or unused segments of the liver obtained from split or reduced grafts. A whole organ perfusion technique was employed to isolate hepatocytes (Mitry et al 2003) and the cells were maintained in an enriched William's E medium as described in section 2.2. The median cell viability of hepatocytes obtained from split/reduced grafts was 70% and of those derived from rejected livers was 35%. Human hepatocytes were used as controls for our *in vivo* and *in vitro* experiments.

2.6.5 Human colon cancer cell line [CaCo-2]:

Growing cultures of CaCo-2 cell lines were purchased from European Collection of Cell Cultures (ECACC, UK) and maintained in a DMEM medium supplemented with additives as described in Appendix 1. The cells were transferred into a T 75 flask and incubated at 37 $^{\circ}$C and 5% CO2. Culture medium was replaced every 3-4 days and flasks were subcultured at 90-95% confluence. Sub-culturing involved removal of culture medium from the flask by aspiration, followed by addition of an appropriate volume of trypsin/EDTA (3ml for a 75ml flask) and incubation at 37 $^{\circ}$C for 3-5 min until cells detached from the flask. Loosely adherent cells were detached by gentle agitation of the flask. The cell suspension was washed twice with culture medium to remove trypsin and finally resuspended in an appropriate volume of culture medium for further culture or preparation of cytospin slides for immunocytochemistry. Cryopreservation of surplus cells was carried out as previously described in section 2.5.4.

2.7 Collagen coating of culture flasks:

Protocol:

Bornstein and Traub Type I calf skin collagen was purchased from Sigma-Aldrich (Sigma C3511) to coat the culture flasks

Stock solution:

1) 0.1M acetic acid is made by dissolving 0.575ml of acetic acid in 99.425ml sterile dH_2O to make a total volume of 100ml.

2) Dissolve collagen in 0.1M acetic acid at 1mg/ml

3) Allow to dissolve over several hours, sterile filter and store in a glass bottle at 4° C

At the time of coating dilute the stock solution at 1 in 20 dilution with distilled water to get a final concentration of 50mcg/ml of collagen.

Coating the flasks:

1) Spread 4ml of diluted collagen solution carefully at the bottom of the flask. Allow the protein to dry in a laminar flow hood for several hours at room temperature

2) Remove the excess fluid from the coated surface and store the flasks at 4° C for future use

3) The coated flasks are washed thrice with PBS before usage

2.8 Morphological, biological and functional assessment of cultured cells:

Cytospin preparation and storage

Detached cells from growing cultures or cryopreserved culture cells that were thawed were washed, and cell counts estimated using a haematocytometer (Merck, UK). The cell pellets were finally resuspended in PBS at a concentration of 10^4cells/ml. After loading cell suspensions onto labelled polyprep glass slides (100µl per spot), cytospin slides were prepared using cytocentrifugation (Hettich, Germany) at 3500 RPM for 6 min. Slides were air dried for 1 hour, and stored in storage boxes at -80^{o}C until use. Pre-prepared cytospin slides with culture cells, Hep-G2, primary HCC or hepatocytes were allowed to thaw at room temperature. The cell spots were circumscribed with a hydrophobic marker pen and the slides were dried for 1hour.

2.8.1 Haematoxylin and Eosin (H&E) staining:

The staining was performed in the following order:

1) Fix section in 10% formaldehyde for 10 minutes
2) Wash in distilled water
3) Stain with Shandon Instant haematoxylin for one minute (The solution was filtered directly onto the slide)
4) Wash in tap water
5) Differentiate nuclei in 1% acid alcohol for 15seconds
6) Wash in tap water until nuclei were blue
7) Counterstain with 1% Eosin for 30 seconds
8) Wash in water and dehydrate the section in absolute alcohol and xylene
9) Mount with synthetic resin DPX

2.8.2 Immunocytochemistry:

Antibodies

All primary antibodies were diluted in 1% PBS; the optimal concentration was determined by serial dilution. Immunocytochemistry was performed using antibodies CAM5.2, Hep Par-1, Polyclonal CEA, Anti human albumin, CD133, CD34, CD90, ATP7b ,CD68 and anti-smooth muscle actin according to the protocols described below

Enzyme Method using Vectastain Streptavidine/Peroxidise kit:

The slides were incubated as follows:

1. 5% formalin for 20 minutes to fix the cells
2. 3% hydrogen peroxide to block the peroxide for 5 minutes (Stock H_20_2 solution = 30% therefore 1:10 dilution = 50µl + 450µl PBS)
3. 10% normal horse serum as blocking serum (100µl normal horse serum+ 900µl PBS) for 30 minutes
4. Primary antibody at 4°C overnight
5. Wash slides with PBS
6. Biotinylated 2° antibody at room temperature for 10 minutes:
   (500µl blocking solution + 2.5µl of universal secondary antibody)
7. Wash slides with PBS
8. Streptavidine/Peroxidase complex for 5 minutes (50 µl of Streptavidine/Peroxidise complex stock solution is added to 2ml of PBS without K^+)
9. Add substrate VIP or DAB (Vector lab Cat SK-4600 or SK-4100) to visualise the staining

10. When the desired colour is achieved the enzyme reaction is stopped by adding PBS
11. Sections were counterstained with haematoxylin for 1min
12. Slides were mounted with 20% aqueous glycerol

Fluorescent method using Vecta fluorescent kit:

1. Slides were incubated with primary antibody overnight following steps 1 and 3 as described in section 3.7.1 (avoid incubation with H2O2)
2. Add anti-mouse/ anti-rabbit fluorescent antibody (25µl of secondary antibody to 1ml of HEPES solution) and incubate at room temperature for 1hour
3. Wash slides with PBS and then with water for 20 seconds
4. Mount the section with propidium iodide containing mounting medium and nuclei counterstaining as red fluorescence

All the incubations were performed in a humidified chamber and were terminated by washing with PBS. The washing procedure was repeated for 3 cycles of 2 min each.

Periodic Acid Schiff (PAS) Staining to detect glycogen:

The staining was performed on the cytospin cell spots as follows:

1. Fix the cells with Carnoy's fixative (100% alcohol 60 ml, chloroform 30 ml and glacial acetic acid 10 ml) for 10 minutes
2. Oxidize in 0.5% periodic acid solution for 5 minutes.
3. Rinse in distilled water.
4. Stain with Schiff reagent for 15 minutes
5. Wash in lukewarm tap water for 5 minutes

6. Counterstain in Mayer's hematoxylin for 1 minute**.**
7. Wash in tap water for 5 minutes.
8. Dehydrate and mount with DPX

2.9 Animal model to assess biology of cultured cells

Experimental animal: Adult 6 week old SCID mice (20–25 g, C.B-17/Icr), purchased from Charles River UK Ltd, were selected as recipients for the cultured disseminated cells. These severely immunodeficient mice have been successfully used as hosts in several tumour implantation experiments. The mice were maintained in filter-cages in the Comparative Biology Centre at King's College London. All animal housing, handling, and experimental procedures were carried out in accordance with the Home Office guidelines for Animal Scientific Procedures UK.

Type of cells:

In vitro cultures from patients with HCC

In vitro cultures from patients with cirrhosis

Human hepatoma cell lines (Hep G2)

Primary healthy human hepatocytes

2.9.1 Cell sample preparation:

Thawing and recovery of cryopreserved cells: The cryo-vials were transferred from liquid nitrogen storage and placed immediately in a 37° C water bath. The nearly thawed cells were diluted 10 times in ice cold culture medium with gentle shaking and cells were purified using a

24% isotonic percoll gradient medium. Following addition of Percoll, the 50ml falcon tube was topped up with culture medium and centrifuged at 250g and 4° C for 20minutes. The supernatant with non-viable cells was discarded and the cell pellet was washed twice with culture medium at 50g for 5minutes.

The thawed and purified experimental and control cell samples were resuspended in culture medium and then transferred into a T25 flask for overnight incubation at 37°C. On the day of transplantation the adherent cells were detached using the cell detachment protocol described in section 2.5.3. Following count and viability check the cells were resuspended in culture medium at a concentration of $2x10^4$cells/ml. One millilitre of this cell suspension was transferred into a sterile eppendoft and micro centrifuged to obtain the final transplantation volume of 50µl.

2.9.2 Cell transplantation:

Anaesthetic technique: 100 µl of hypnorm and 100 µl of midazolam was diluted twice with distilled water, 60 µl of this diluted mixture was injected into the intraperitoneal cavity of each mice.

Liver injections: The anaesthetised mice were placed on a warm operating surface and the ventral surface was shaved and sterilised using 100% ethanol. The abdominal cavity was accessed by a 1.5 cm midline incision, small bowel was retracted using a cotton swab and the liver was exposed. The prepared 50µl cell suspension with 20000 cells was injected directly into left lobe of the liver using a Hamilton micro syringe and the incision was closed with interrupted vicryl sutures.

Kidney injections: The abdominal cavity of the anaesthetised mice was opened by a 1 cm incision below the left costal margin. The lower pole of the kidney was clearly visualised after retracting the small bowel. The prepared cell volume was injected into the middle of the lower pole margin and the abdomen was closed with interrupted vicryl sutures.

Following injections the mice were labelled by a punch excision of the anterior or posterior surface of the right or left ear lobe and were transferred into their cages.

2.9.3 Retrieval of transplanted cells:

Retrieving the transplanted liver: Mice were sacrificed six weeks after transplantation by dislocating the cervical vertebrae. The liver was retrieved in total and the cell transplant segment was excised separate from the whole organ. The individual portions were transported to the laboratory in ice cold UW solution and stored in labelled cryo-vials at -80°C following snap freezing.

Retrieving transplanted kidney: The mice were sacrificed two weeks following cell transplantation. Both the kidneys were retrieved in total and the left kidney was divided transversely to obtain separate upper and lower poles. The two halves of the left kidney and the whole right kidney were snap frozen and stored in separate cryo-vials at -80°C further analysis.

2.9.4 Tissue sections for analysis:

The samples were either processed and embedded in paraffin wax or the frozen tissue was directly sliced into 10 μm cryosections.

The paraffin sections were dewaxed by immersion in xylene followed by absolute alcohol. H&E staining was performed on the frozen and deparaffinized tissue slides using the protocol described in section 2.8.1.

Immunohistochemistry on paraffin sections:

Following dewaxing the slides were rinsed in distilled water and phosphate buffered saline. Antigen retrieval was performed by immersing the slides in a 0.1M citrate buffer at pH 6.0 and heating in a microwave at 700 watts for 25 minutes. After leaving to cool for 20 minutes at 4°C the slides were washed in PBS and sections were circumscribed with a hydrophobic marker pen.

The Novacastra ready-to-use peroxidase detection system was used in the following order for enzyme staining. All steps were performed in a humidified chamber at room temperature and the incubations were terminated by PBS wash (3 cycles of 2 minutes each).

1. Neutralize endogenous peroxidase using peroxidase block for 5 minutes
2. Incubate with protein block for 5 minutes
3. Incubate with optimally diluted primary antibody for 60 minutes
4. Incubate with biotinylated secondary antibody for 30 minutes
5. Incubate with streptavidin-HRP polymerase for 30 minutes
6. Develop peroxidase activity with a freshly prepared DAB working solution (50µl of DAB Chromogen to 1ml of DAB substrate buffer) for 5 minutes
7. Counterstain with Hematoxylin
8. Mount sections with glycerine

The techniques described in section 2.7.2 were used for frozen sections

Positive and negative controls: All of the immunocyto/immunohistochemical analysis experiments were performed using positive and negative controls. Two types of negative controls were used: one was by performing all the steps in the protocol but omitting the primary antibody and the other was staining a known negative sample.

The tissue sections were analysed using cytokeratin 8 and 18, MHC class 1, Ki 67 and CD34 antibodies in optimal dilutions. To further support the presence of transplanted human cells, the tissue specimen was analysed for the presence of human genomic DNA.

2.9.5 Molecular techniques:

Total RNA extraction: Total RNA was extracted using Trizol reagent (Invitrogen). Briefly, cells were lysed by pipetting Trizol in a 1.5ml sterile eppendoft tube (approximately 800µl per 10^4 cells) and incubated for 5 min at room temperature. Chilled chloroform was then added (0.2ml per 1ml Trizol) and after shaking vigorously for 15 seconds the tube was incubated for 3 min at room temperature (RT). After centrifuging at 12000 x *g* for 15 min and 4°C, the aqueous phase supernatant containing total RNA was transferred to a sterile eppendoft tube containing an equal volume of isopropanol and incubated for 10 min at RT. RNA was precipitated by centrifugation (12000g, 4^oC, 10 min) and washed carefully with 75% ethanol (1ml of ethanol for 1ml of trizol reagent at 7500g, 4^oC, 5min). The RNA pellet was allowed to dry and dissolved in 5-30µl of RNase-free water and stored at -80^oC. The purity of RNA samples was assessed by measuring the OD260/OD280 ratios on a NanoDrop ND-1000 spectrometer (NanoDrop, Wilmington, DE), resulting in a ratio of 2.00 - 2.08 in all cases.

DNA isolation: After complete removal of the aqueous phase containing RNA, the interphase and organic phase were incubated with 100% ethanol (0.3ml of ethanol per 1ml of Trizol) for 2 min at RT and DNA was precipitated by centrifugation at 2000g for 5minutes and $4^{o}C$. The DNA pellet was washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol (1ml of solution per 1ml of Trizol at 2000g, $4^{o}C$, 5min), resuspended in 75% ethanol for 20 minutes at RT and centrifuged at 2000g, $4^{o}C$, 5minutes. The DNA pellet was allowed to dry and dissolved in a pH adjusted 8mM NaOH solution for long term storage at $4^{o}C$.

DNA isolation from paraffin blocks: A total nucleic acid isolation kit for paraffin embedded tissues (Invitrogen) was used as per the company protocol. After deparaffinization and rehydration with 100% ethanol the tissues were incubated with digestion buffer (100µl of buffer for ≤ 40µm and 200 µl for 40-80 µm of tissue) at $50^{o}C$ for 16 hours. Following addition of an appropriate amount of isolation additive and ethanol (for 100 µl of digestive buffer -120 µl of isolation additive and 275 µl of 100% ethanol) the mixture was passed through a filter cartridge and washed with 700 µl of wash 1 and 500 µl of wash 2. 60 µl of RNase mix (10 µl of RNase and 50 µl of nuclease free water) was added to the filter cartridge and incubated for 30min at RT. After washing and centrifugation at 10000g for 30 seconds the filter cartridges are transferred onto fresh collecting tubes and 60 µl of nuclease free water was applied to collect the isolated DNA.

Micro array:

Sample processing for Human Gene 1.0 ST arrays

Total RNA was extracted using Trizol reagent (Invitrogen) and quality/integrity was assessed using ribosomal RNA band analysis on a 2100 Bioanalyser and RNA 6000 Nano LabChips

(Agilent). 75ng of total RNA was reverse transcribed and amplified into cDNA using NuGEN's Pico WT–Ovation labelling kit following the manufacturer's protocols (NuGEN Inc.). The Exon conversion module from NuGEN was used to synthesise, the sense orientation copy from amplified cDNA and the Biotin module for biotin-labelling. We followed NuGEN's recommendations for Affymetrix Human Gene 1.0 ST arrays (Affymetrix, Santa Clara) and subsequent processing using standard hybridisation, washing and staining reagents (Hybridisation Wash Stain (HWS) kit). Scanned array images (DAT and CEL files) were generated using Affymetrix's AGCC software and analysed using their Expression Console package which generates normalised and background-corrected probeset-summarised signals for each gene on the array. The standard gene-level RMA workflow was used to achieve this data output. Control probeset data was removed from the main dataset prior to data analysis proper. To do this we deleted the rows which contained information for various 'normgene' probesets (as shown in the 'category' annotation column of the whole data table). This resulted in removal of 4201 probesets from the original summarised dataset (33,297 probesets) leaving 29,096 probesets for further analyses.

Data analysis:

The filtered data table was formatted as a '.gedata' tab-delimited text file and imported into Qlucore's Omics Explorer 2.1 software for analysis. The software, which utilises a visual, Principal Components Analysis (PCA) approach to display the relationships between samples and genes allowed the selection of differentially expressed genes using standard statistical techniques. We employed a simple 1-way ANOVA to filter genes which were differentially regulated across the different sample groups (bone marrow CD34+cells, HCC cells, HepG2 cells and primary hepatocytes), and used the p-value slide bar to create the various statistical cut-off gene lists for the different comparisons of interest. Gene lists (containing all regulated genes) were displayed as heat maps to show gene expression patterns within the list, and sub-lists of interest were selected on the basis of specific expression patterns.

Real time RT-qPCR:

We used a single-step real-time PCR with the 7900HT Fast Real-Time PCR System (Applied Biosystems Inc, CA, USA). RNA was reverse transcribed using TaqMan® Gold RT-PCR Kit one step reaction mix. qPCR was performed in 20-µl reaction mixtures using TaqMan gene expression master Mix (Applied Biosystems) in 96 well plates. The mix was optimized for real-time PCR quantitative analysis and contained AmpliTaq Gold® DNA Polymerase UP (Ultra Pure), Uracil-DNA Glycosylase, dTNPs with dUTP, Passive Reference 1 and optimized mix components of 6 primers (Glypican, Lyve1, Survivin). The thermal cycling conditions comprised an initial 2-minute, 50 °C step for optimal UDG enzyme activity and a 10-minute, 95 °C to activate the AmpliTaq Gold UP enzyme followed by 40 cycles of 95°C for 15 seconds(denature), and 65°C for 1 minute(anneal/extend). An external standard template containing human GAPDH cDNA was included in each run to generate a standard curve.

PCR:

The PCR selection kit (Invitrogen) was used according to the company protocol to amplify the relevant DNA samples. The reaction mix consisted of PCR buffer, dNTP mixture, $MgSO_4$, Primer mix, Template DNA, Platinum® Taq and autoclaved distilled water to make a final concentration of 50µl. The tubes were incubated in a thermal cycler at 94°C for 2 minute to completely denature the template and activate the enzyme. 40 cycles of amplification was performed at 94°C for 15–30 seconds (denature), 55°C for 15–30 seconds (anneal) and 68°C for 1 minute (extend).

2.10 Slide interpretation

The stained slides were visualised under confocal or fluorescent microscopy as necessary. The experimental slides were examined after positive and negative control slides to ensure successful staining. The morphology of cells and pattern of staining was critically examined in the experimental samples before deeming them positive. All results were confirmed by an expert liver histopathologist who was blinded to avoid bias.

CHAPTER 3: ISOLATION AND *IN VITRO* CULTURE OF DISSEMINATED CELLS

3.1 Introduction: Hepatocellular carcinoma (HCC) is emerging as a major health issue in the Western world. It is currently the leading cause of death among patients with chronic liver disease (Llovet 2005). Surgical resection and transplantation can achieve best outcomes in well-selected patients (5-year survival of 60%–80%) and are offered as the first line curative procedures. Tumour recurrence is a major obstacle following surgical resection and also complicates liver transplantation despite the use of strict selection criteria. The outcomes of patients with recurrence are poor and currently it is a significant cause of late death (Fong 2004). Recurrence can be explained by three different mechanisms; inadequate tumour resection, intrahepatic haematogenous or lymphatic tumour cell spread, and de novo (multicentric) tumor development in a cirrhotic liver (Schlitt 1999). However tumour recurrence in patients following radical total hepatectomy and liver transplantation has raised the possibilities of disseminated microscopic tumour cells (DTC) or an extrahepatic origin of HCC.

Tumour size and number are currently the only surrogate markers available to select patients and unfortunately these are not reliable determinants of tumour biology (Chen 2009). Additional markers that could improve the current staging systems need to be explored. The correlation between the presence of DTC and tumour recurrence has led to the development of techniques to identify these cells in several epithelial cancers but still knowledge on their biological behaviour is unknown. Pre-operative detection of these DTCs may help to identify patients in need of additional systemic therapies after curative resection of primary tumour.

Several studies have described immunocytochemical and molecular assays to detect DTC in regional lymph nodes, peripheral blood and bone marrow of patients with epithelial tumours including HCC. The current understanding of DTC and their relationship to tumour recurrence is largely derived from studies on breast cancer. Experience from breast cancer suggests that the mere presence of DTC does not predict the risk of metastases and additional molecular characterization is required to assess their biological potential. It has been shown that DTC in breast cancer can remain dormant for prolonged periods, are resistant to chemotherapy and have a cancer stem-cell phenotype(CD44+CD24–/low,cytokeratin19+muC1–,epCam+) suggesting a role for cancer stem cells in the propagation of DTC (Pantel 2009).

The primary aim of this chapter was to develop a protocol to isolate and perform *in vitro* expansion of disseminated tumour cells in patients with HCC. The secondary aim was to assess the morphological and functional characteristics and expression of stem cell like phenotype of the cultured cells using immunocytochemistry.

3.2 Patients and methods:

During the study period a total of 45 patients with hepatocellular carcinoma provided written consent to participate. One patient subsequently withdrew and therefore peripheral blood samples were collected from 44 patients (Group 1). The median age was 61 years (Range 26-82 years) and 84% of the patients were male. The aetiology of underlying liver disease was either alcohol induced or hepatitis viral infection in the majority. The median tumour size was 3.4 cm (Range 1.3-17cm). Macrovascular invasion was noted on the pre-operative staging investigations of 4 patients (9%). The median MELD score at presentation was 10 (Range 6-20).

The control group consisted of 50 patients with chronic liver disease, but without hepatocellular carcinoma (Group 2- liver disease controls). The median age was 52 years (Range 19-67 years) and 30% of this group were of female sex. The aetiology of liver disease was alcohol related in 45% and the rest comprised of a mixture of hepatitis infection, cholestatic liver diseases and cryptogenic cirrhosis. The median MELD score at presentation was 14 (Range 7-34). The aetiology of liver disease in the patient and control groups is summarised in Table 1.

Table 1: Aetiology of underlying liver disease

Aetiology of liver disease	Patients with HCC (n=44)	Patients with cirrhosis but without HCC (n=50)
Alcohol related chronic liver disease	20	22
Viral (HBV and HBC)	20	10
Auto-immune	1	5
Cholestatic liver disease(PBC&PSC)	2	6
Cryptogenic	1	3
Metabolic (enzyme deficiencies, NASH)	-	4

Twenty normal subjects with neither chronic liver disease nor hepatocellular carcinoma were also included in the study (Group 3- healthy controls). The median age was 32years (Range 21-45 years) and majority were of female sex (73%).

Establishing an in vitro culture protocol:

There is no published evidence regarding successful culture of DTC from HCC. The experience with primary *in vitro* expansion of DTC from other epithelial cancers is very limited. Initial experiments with breast cancer micrometastases revealed that these cells have a time-limited proliferative potential and therefore expansion of cell number is difficult. With this experience Pantel et al focused on the development of an immortalized micrometastatic cell line with a process that leads to the selection of only those cell clones which adapt best to the culture conditions (Pantel 1999).

The high-glucose Dulbecco's modified eagle medium (D-MEM) with 15% fetal bovine serum (FBS) described by Loo et al (2005) for primary culture of DTC was used initially with our patient samples. Unfortunately this medium did not support the growth of DTC from HCC patients.

The isolation and expansion of cells is strongly associated with the culture conditions. Modifications to the culture medium, cell density or culture flasks can make significant changes to cell growth. In an attempt to establish a culture protocol for the cells of interest, changes were made to the type of medium (DMEM vs. Alpha MEM), cell density (T25cm2 vs. T75cm2 culture flasks) and culture flasks (collagen coated vs. non-coated flasks).

Growth factors regulate cell replication by providing both stimulatory and inhibitory signals for proliferation. Their use has been described in several *in vitro* experiments at optimal levels to enhance cell proliferation. A variety of growth factors have potent mitogenic effects on Recombinant hepatocyte growth factor (HGF), a potent mitogen first purified from rat platelet and human and rabbit plasma (Nakamura 1986, 1987, 1989) has been studied extensively with respect to DNA synthesis and proliferation *in vitro* (Richman 1976; McGowan 1981). HGF stimulates hepatocyte DNA synthesis and proliferation within 3 to 4 hours of its addition to the culture flask (Kimura 1997). The role of using HGF to differentiate bone marrow progenitor cells into functional hepatocytes has been described by Jiang et al (2002).

Alpha- minimum essential medium (MEM) was described to be not only the optimum medium but also superior to DMEM for the expansion of hematopoietic cell lineage (Sotiropoulou 2006, Chen 2009). An enriched Alpha-MEM with 20% FBS and recombinant hepatocyte growth factor to favour the growth of possible hepatocyte like cells was therefore selected as the medium for expansion of our cells.

The mononuclear cell layer (Buffy coat) from the blood specimen was isolated by density centrifugation and the potential DTC were cultured using the protocol described in Chapter 2. The medium was replaced on alternate days and culture flasks were monitored every day for cell attachment, viability and growth. The culture was carried on for a minimum of two weeks to allow growth of disseminated cells. The established cell colonies were detached and cytospin slides for immunocytochemical analysis were prepared using the protocols described in chapter 2.

Antibodies to assess the morphology of cultured cells:

Cytokeratin 8 and 18(Cam 5.2): The cytokeratins form a cytoplasmic intermediate filament network in many epithelial cells. There are approximately 20 different polypeptides, whose expression patterns differ among different epithelial tissues. Normal adult human hepatocytes express cytokeratin 8 and 18. These filaments form a pericanalicular sheath that envelopes the whole hepatocyte resulting in a membranous immunocytochemical staining (Su 1998).

Hepatocyte paraffin -1 (Hep Par-1): Hepatocyte specific antigen (HSA) is expressed by the majority of HCC's and normal hepatocytes and not by biliary or non-parenchymal liver tissue. HSA binds specifically to a mouse monoclonal antibody, Hepatocyte Paraffin-1 (Hep Par-1) which is used to differentiate primary hepatocellular tumours from tumours of biliary or metastatic origin in cases of diagnostic uncertainty (Wennerberg 1993, Leong 1998, Siddidui 2001, Zimmerman 2001). The exact location of the HSA antigen is unclear but it is thought that the epitope is located in hepatocellular mitochondria which results in a granular cytoplasmic immunocytochemical staining. Hep Par-1 has been established as a reasonably sensitive marker for HCC (Lau 2002).

Polyclonal Carcinoembryonic antigen (PCEA): CEA is an oncofetal glycoprotein that is present in small amounts in normal adult gastrointestinal, pancreatic, and biliary tract cells. A characteristic biliary canalicular pattern of PCEA expression is observed in majority of HCC lesions. This pattern of PCEA immunoreactivity is a result of cross-reactivity with biliary glycoprotein 1 that is expressed by normal bile ducts and bile canaliculi. The reported sensitivity of PCEA staining for HCC ranges from 60-95% (Borscheri 2001). Normal liver also exhibits a canalicular staining pattern with this antibody (Porcell 2000).

ATP 7b: Is a transporting P-type ATPase that plays a key role in copper metabolism. Wilson's disease gene (ATP 7b) that codes for this protein is primarily expressed in the liver and kidney. The protein is localized to the plasma membrane of both hepatocytes and biliary epithelial cells and helps transport copper into bile and serum as ceruloplasmin. Mutation of ATP 7b gene is the cause of Wilson's disease that results in excessive deposition of copper mainly in the liver and brain. ATP 7b antibody was used in our study to assess the expression of hepatocyte like phenotypes by the cultured cells.

CD68: Cluster of differentiation 68 is a 110-Kd transmembrane glycoprotein of unknown function highly expressed by human monocytes and tissue macrophages.

Anti SMA: Smooth Muscle Actin (SMA) is found in the cytoplasm of smooth muscle cells. The anti-SMA antibody stains Kupffer cells in the liver, smooth muscle cells of blood vessels, and gastrointestinal tract.

Both CD 68 and Anti-SMA were used to exclude the presence of non-hepatocyte like cells in the cultured colonies.

Antibodies to assess the expression of stem cell like phenotype:

CD34: CD 34 is an adhesion molecule with a putative role in early haematopoiesis by mediating the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells. It is highly expressed on haematopoietic progenitors, as well as on endothelial cells, brain, and testis.

CD133: This was originally known as AC133 and is a member of pentaspan transmembrane glycoprotein family. CD133 antigen is a cell surface marker mainly expressed by haematopoietic stem cells but its function is currently unknown. It is also expressed by neural and endothelial stem cells, foetal liver and some malignant tumours.

CD90: It is a cell membrane bound glycoprotein also known as Thy-1 antigen. CD90 is expressed by a variety of progenitor cells such as, haematopoietic stem cells, mesenchymal stem cells, hepatic stem cells and keratinocyte stem cells. The function of CD 90 is yet not fully elucidated, however it is thought to have roles in apoptosis, cell adhesion, cell migration and tumour metastases (Abeysinghe 2003).

The expression of hepatocyte specific characteristics by the *in vitro* cultured cells was further assessed using the following functional markers:

Human albumin: Albumin is the most common protein in the serum and is produced by the liver. A commercially available anti-human albumin antibody was used to analyse the cultured cells.

Glycogen: Also known as animal starch serves as long-term energy storage in the body. It is primarily synthesized and stored in hepatocytes and muscle cells. The presence of glycogen in the cultured cells was assessed by using PAS stain.

The immunocytochemical analysis for the relevant antigens and PAS stain to assess the presence of glycogen was performed as per the protocols described in chapter 2.

3.3 Results:

***In vitro* Culture:**

Peripheral blood was cultured from subjects in group 1 (Patients with HCC), group 2 (chronic liver disease controls) and group 3 (healthy human volunteers) under identical conditions. Robust colony growth, as evidenced by cell counts in excess of $3x10^4$ was noted in 6 patients from group 1. The cell colonies were widely separated and consisted of closely arranged groups of oval/round cells with a prominent centrally located nucleus as shown in Figure 1. Small colonies of large cells ranging from 5000 to 10000 in number were noted in 15 patients from group 1 and 5 patients from group 2 (Figure 2). No cell growth was noted in controls from group 3 at the end of a two-week culture (Figure 3). The cells were slow growing and no significant difference was noted in the external morphology between cells cultured from group 1 and group 2 when reviewed by an expert liver histopathologist (Figure 4). Collagen coating of the culture flasks, changes to cell density or prolongation of culture period did not alter cell attachment or growth.

Figure 1: Robust cell growth - cell colonies of oval/round cells with prominent central nucleus (Day 10 *In vitro* culture)

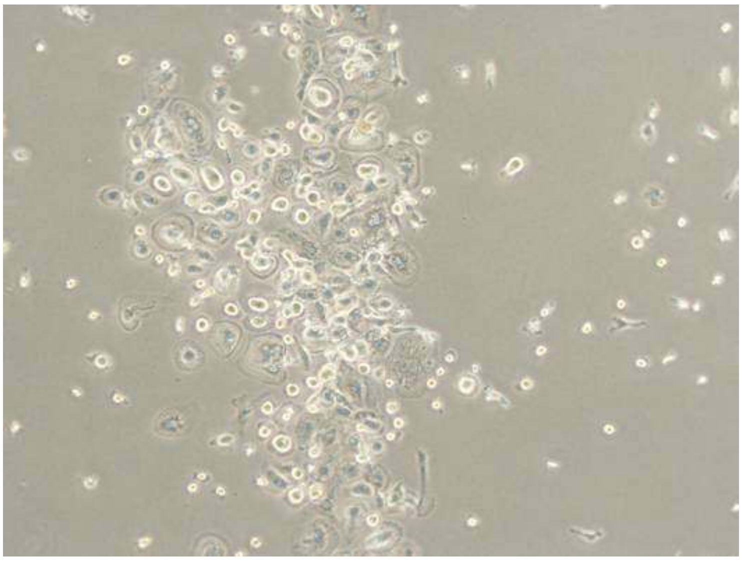


Figure 2: Unsustained growth- small colonies of oval/round cells with prominent central nucleus

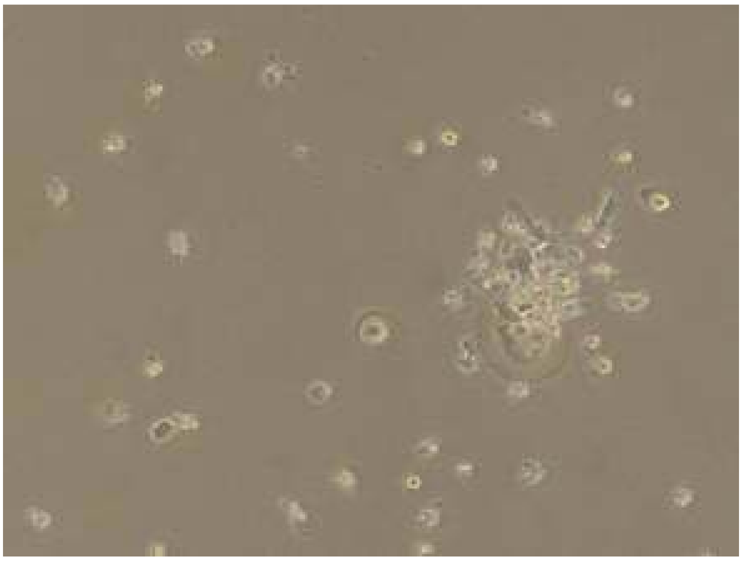


Figure 3: *In vitro* culture- peripheral blood samples with no cell growth

Figure 4:

Cells from patients with cirrhosis but no HCC

Cells from patients with cirrhosis and HCC

The cell colonies were detached washed and analysed using immunocytochemistry.

Cell morphology: Detached cultured cells from groups 1 and 2 stained were first stained with H&E to study the external morphology. This revealed large hexagonal cells with a peripherally placed nucleus resembling the appearance of hepatocytes as shown in Figure 5.

Negative staining with CD 68 and Anti SMA antibodies excluded the possibility of these large cells being macrophages or Kupffer cells (Figure 6&7).

Further analysis of the morphology of cultured cells using Cam 5.2, Hep Par-1, Polyclonal CEA and ATP 7b showed features similar to human hepatocytes. The relevant pictures along with the corresponding positive and negative controls are shown below in Figures 8,9,10 and 11.

Functional phenotype: Cultured cells from both HCC and cirrhotic controls expressed albumin and glycogen which are characteristic markers of hepatocellular function (Figures 12 and 13).

Stem cell phenotype: A significant proportion of cultured cells from group 1 and 2 expressed CD34, however, the expression of CD133 and CD90 was sparse and confined only to cultured cells from patients with HCC (Figures 14, 15 & 16)

Figure 5a) Human hepatocytes isolated for hepatocyte transplantation: H&E staining demonstrating a population of uni or bi nucleate hexagonal cells.

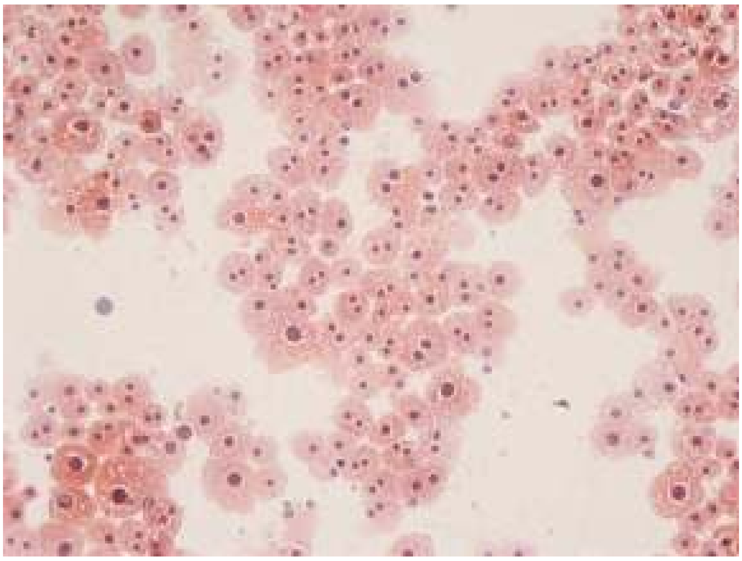


Figure 5b) Cultured cells from patients with cirrhosis and HCC: H&E staining showing hexagonal cells with peripherally placed nucleus resembling hepatocytes.

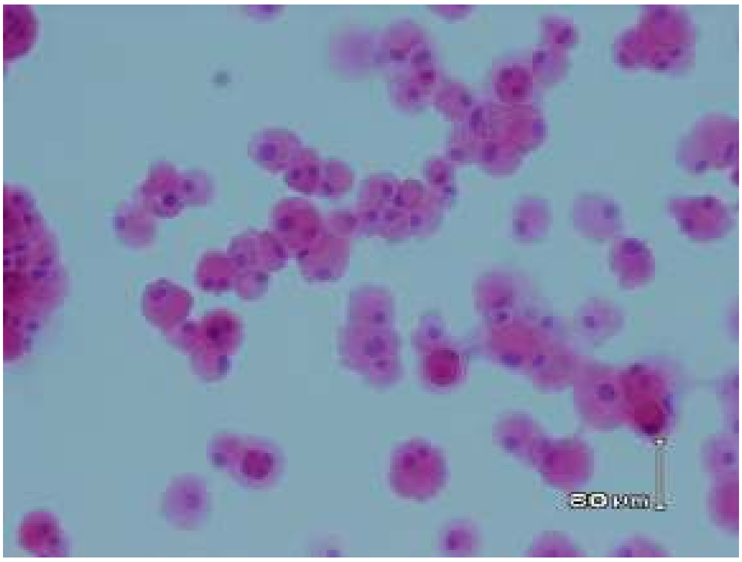


Figure 5c) Cultured cells from patients with cirrhosis but no HCC: H&E staining showing hexagonal cells with peripherally placed nucleus resembling hepatocytes.

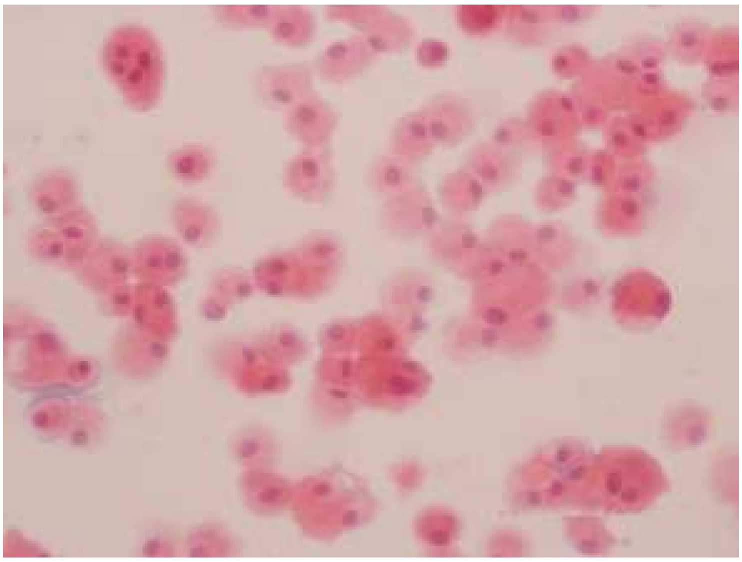


Figure 6a) Negative staining with CD 68 antibody excluding the possibility of *in vitro* cultured cells being macrophages or Kupffer cells

Figure 6b) Immunocytochemistry using CD 68 antibody (Positive control): Human small bowel tissue macrophages

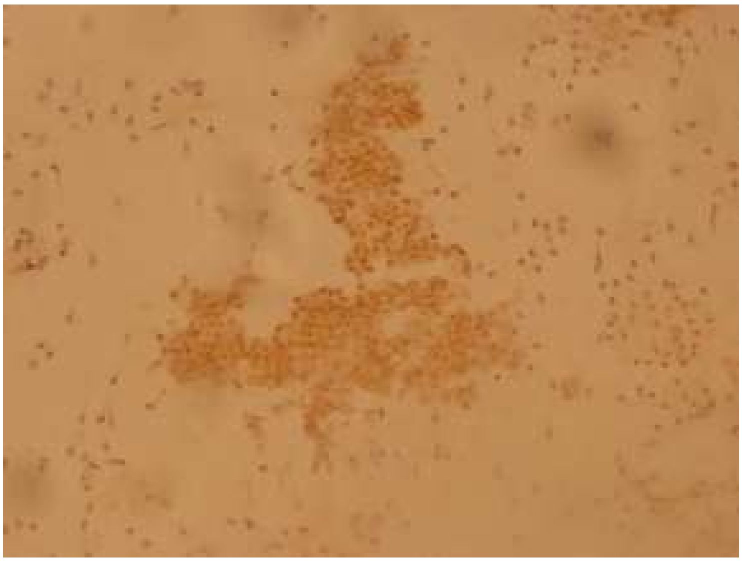


Figure 7a: Immunocytochemistry using anti SMA antibody: Negative staining excluding the possibility of *in vitro* cultured cells being macrophages or Kupffer cells

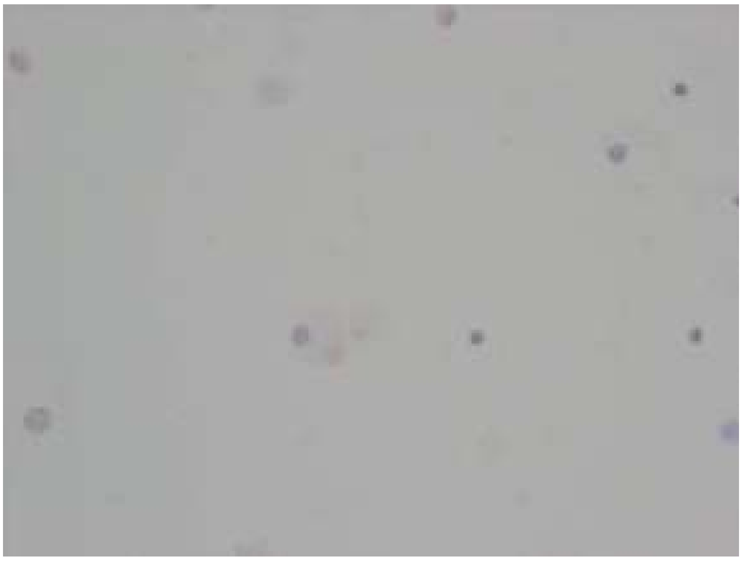


Figure 7b: Immunocytochemistry using anti SMA antibody (positive control): Human intestinal smooth muscle cells.

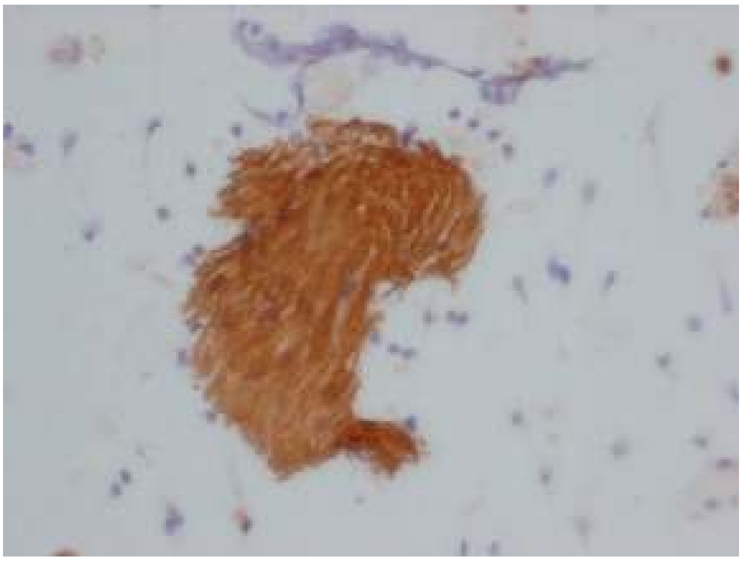
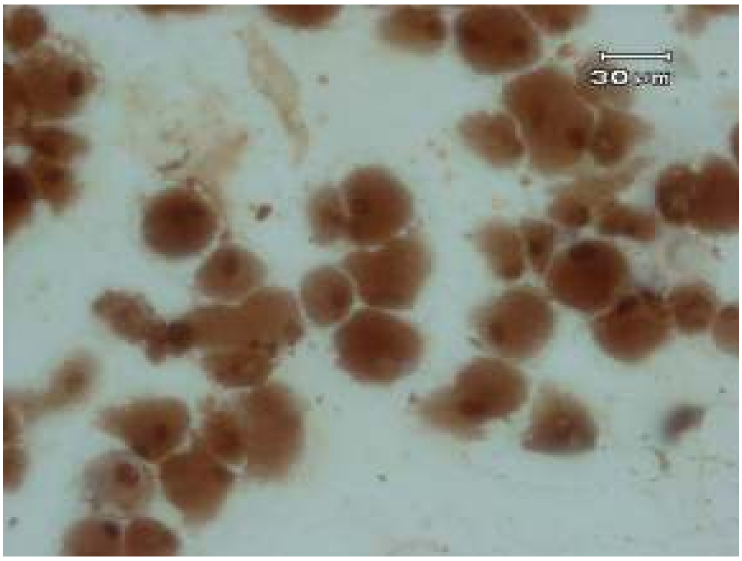
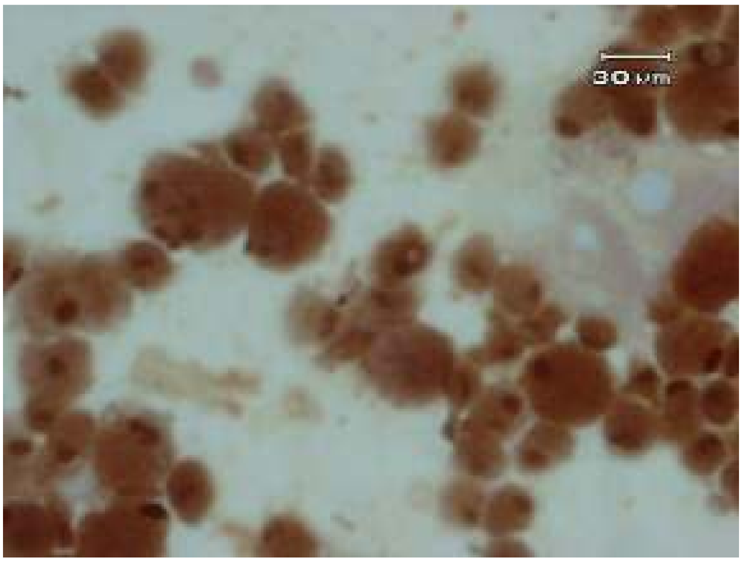


Figure 8: Immunocytochemistry using antibody against cytokeratin 8&18 (Cam 5.2):

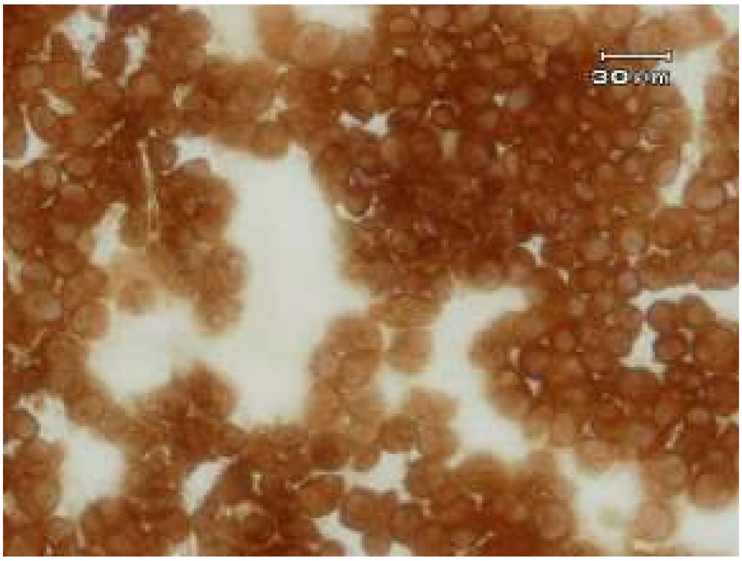
8 a) Cultured cells from patients with cirrhosis and HCC: The cells demonstrate a diffusely positive cytoplasmic staining with cam 5.2 antibody



8 b) Cultured cells from patients with cirrhosis but without HCC: These cells also expressed cytokeratin markers 8&18 as shown by the positive cytoplasmic staining with cam 5.2 antibody.



8 c) Positive control- Human hepatoma cell line (HepG2)



8 d) Negative control- Murine pancreatic beta cell line (MIN): The nuclei were counterstained with haematoxylin

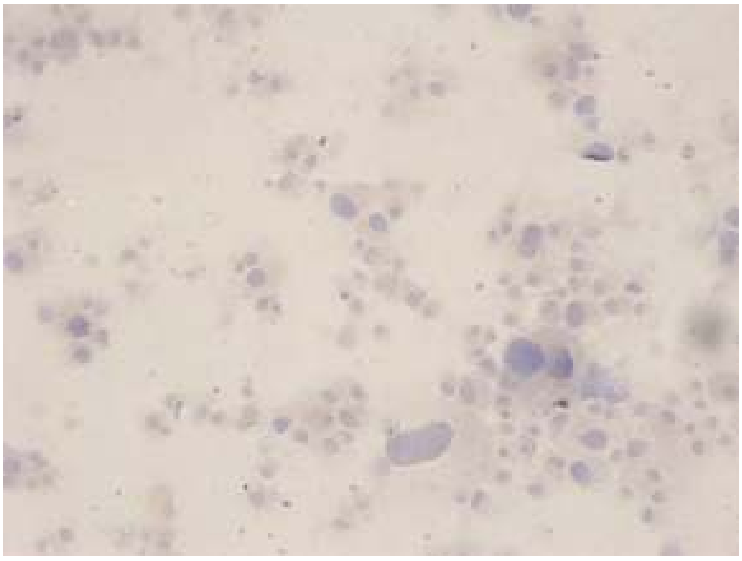
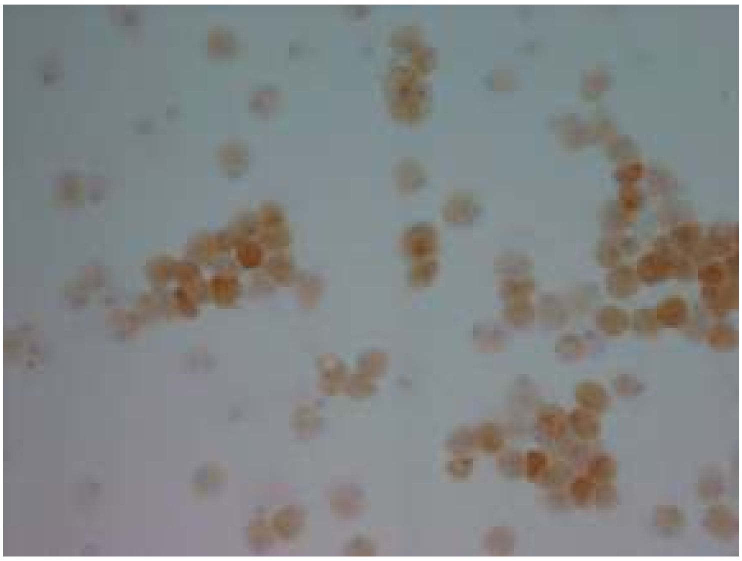
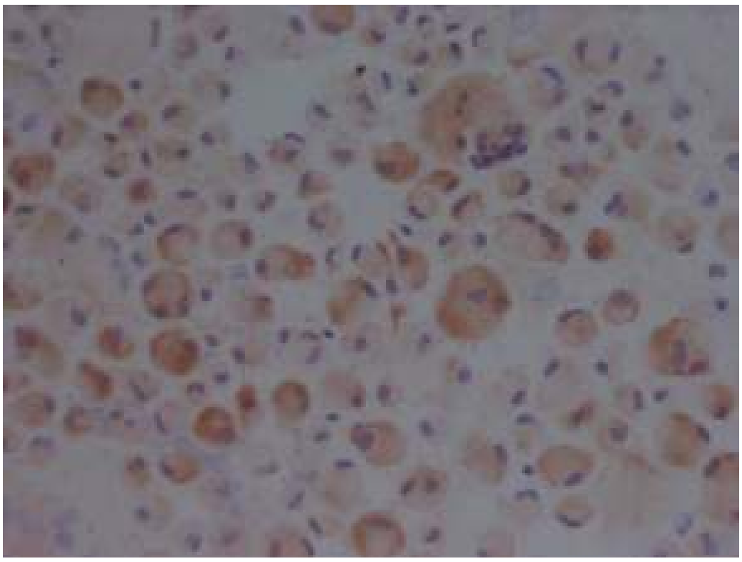


Figure 9: Immunocytochemistry with antibody against hepatocyte specific antigen (HSA) - Hep Par-1:

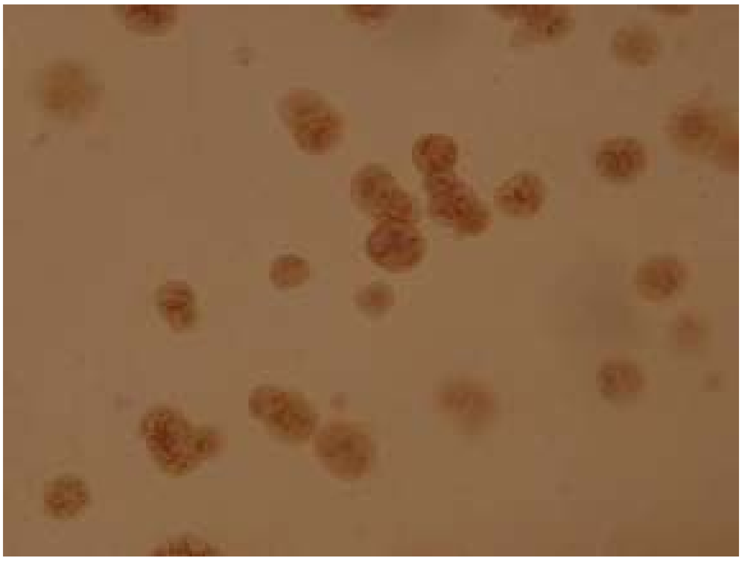
9 a) Cultured cells from patients with cirrhosis and HCC: Staining is characteristically cytoplasmic, the nuclei were counterstained with haematoxylin.



9 b) *In vitro* culture cells from patients with cirrhosis but no HCC: Positive cytoplasmic staining demonstrating the expression of HSA similar to the cells cultured from patients with cirrhosis and HCC



9 c) Positive control- Normal human hepatocytes



9 d) Negative control-Murine pancreatic beta cell line (MIN) - Nuclei counterstained with haematoxylin

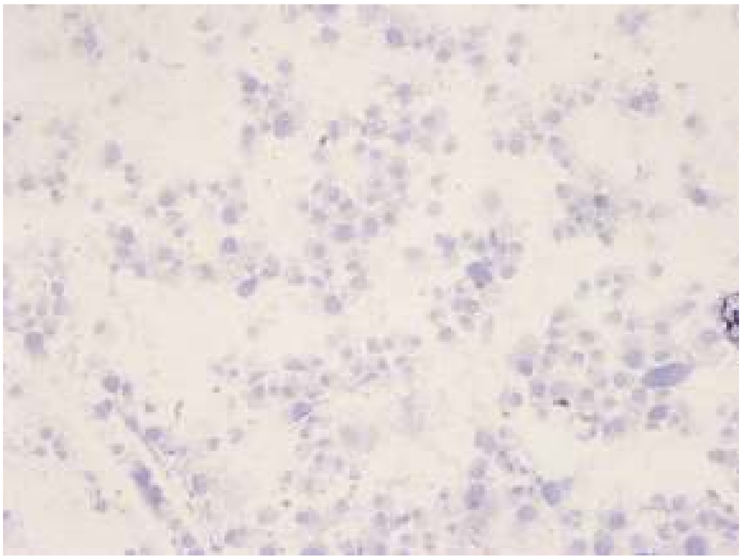
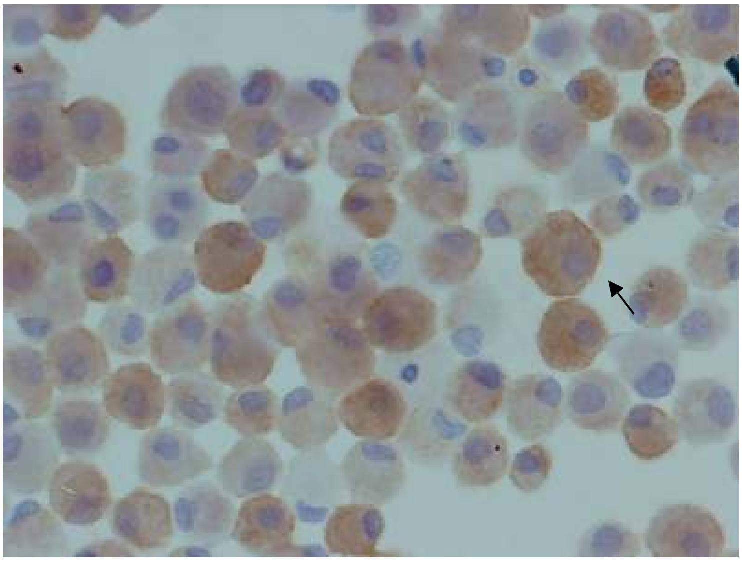
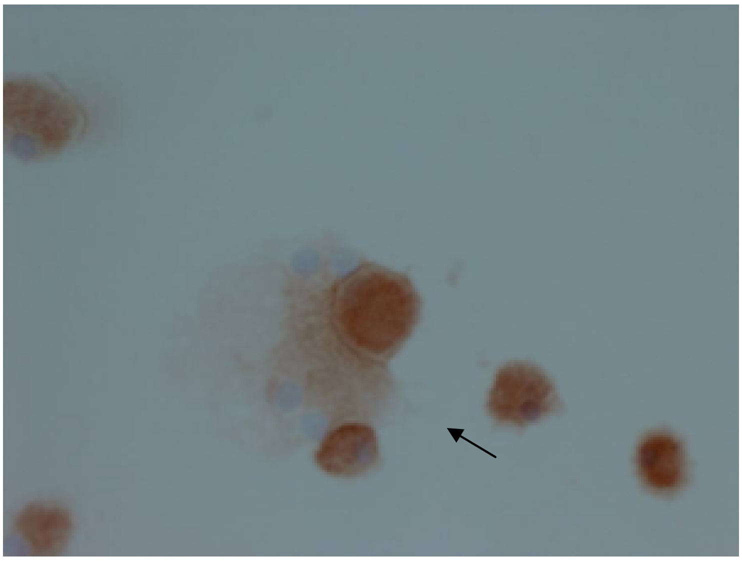


Figure 10: Immunocytochemistry with antibody against Polyclonal CEA:

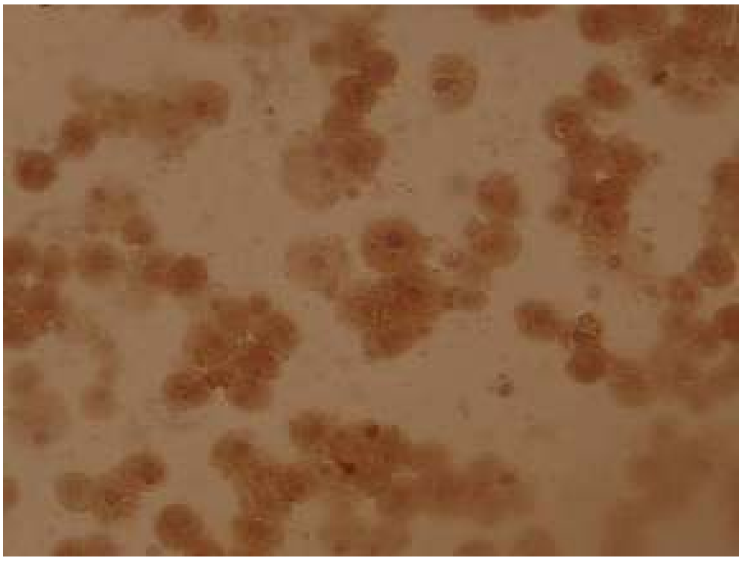
10 a) Cultured cells from patients with cirrhosis and HCC: A characteristic biliary canalicular pattern of immunocytochemical staining (arrow) was noted with this antibody



10 b) Cells cultured from patients with cirrhosis and without HCC: These cells also expressed PCEA as shown by the biliary canalicular pattern of staining (arrow)



10 c) Positive control- Normal human hepatocytes



10 d) Negative control- Murine pancreatic beta cell line (MIN) - Nuclei counterstained with haematoxylin

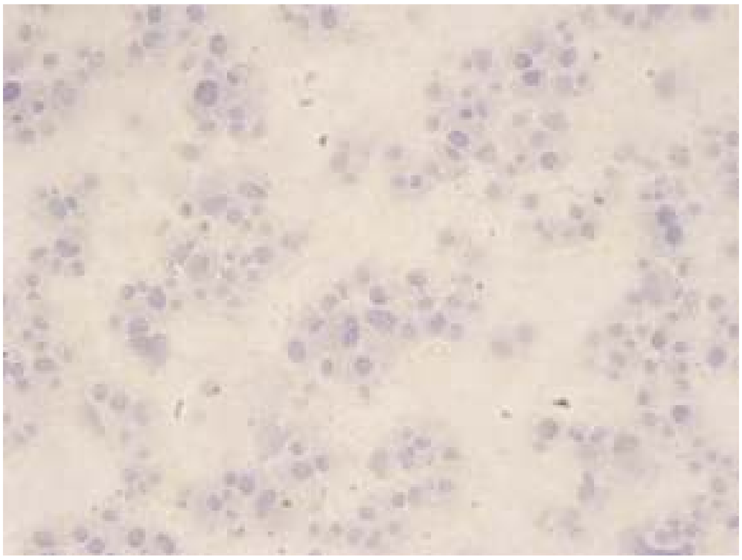
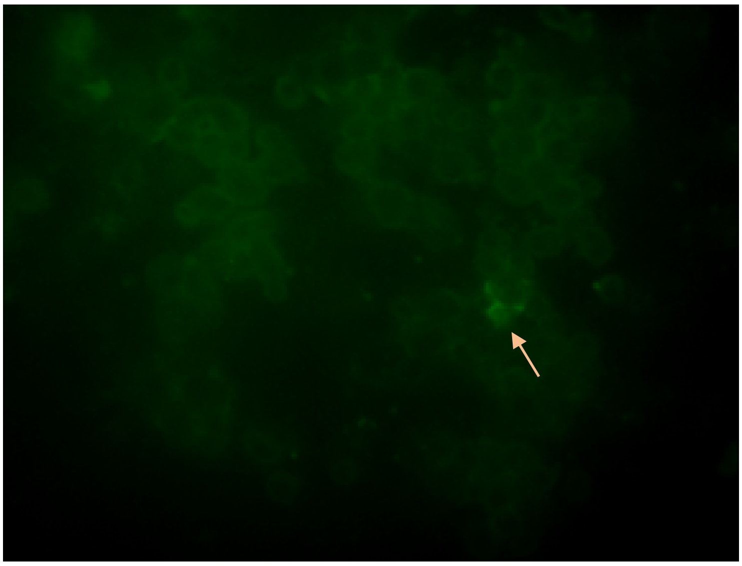
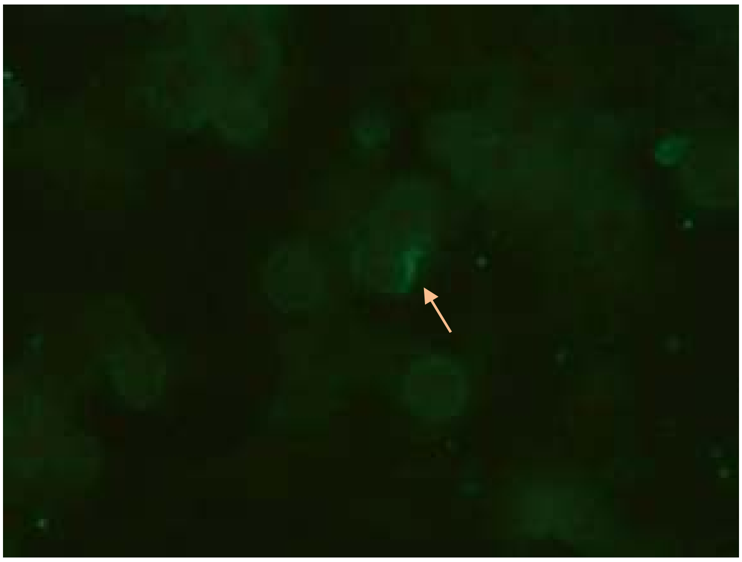


Figure 11: Immunocytochemistry with anti ATP 7b antibody:

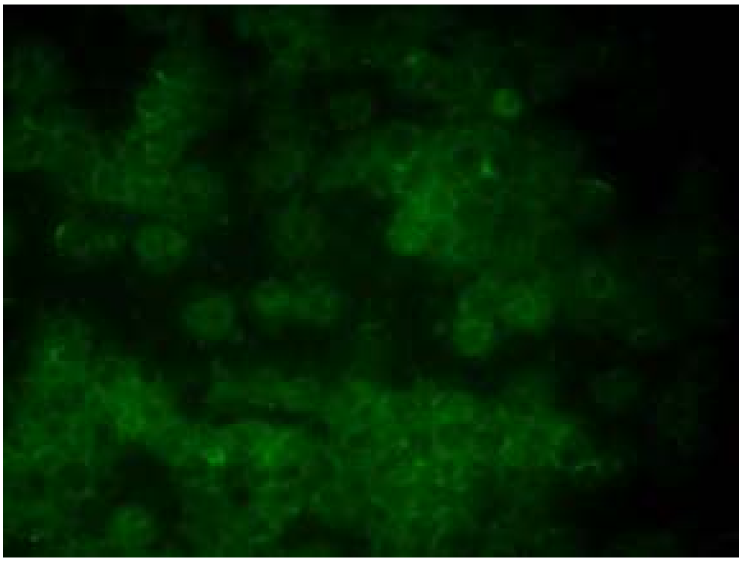
11 a) Cultured cells from patients with cirrhosis and HCC: A typical membrane staining was noted as shown by the marker.



11 b) *In vitro* culture cells from patients with cirrhosis but no HCC: Membrane staining was noted with anti ATP 7b antibody (arrow)



11 c) Positive control- Healthy human hepatocytes.



11 d) Negative control- Human colon cancer cell line (Caco2)

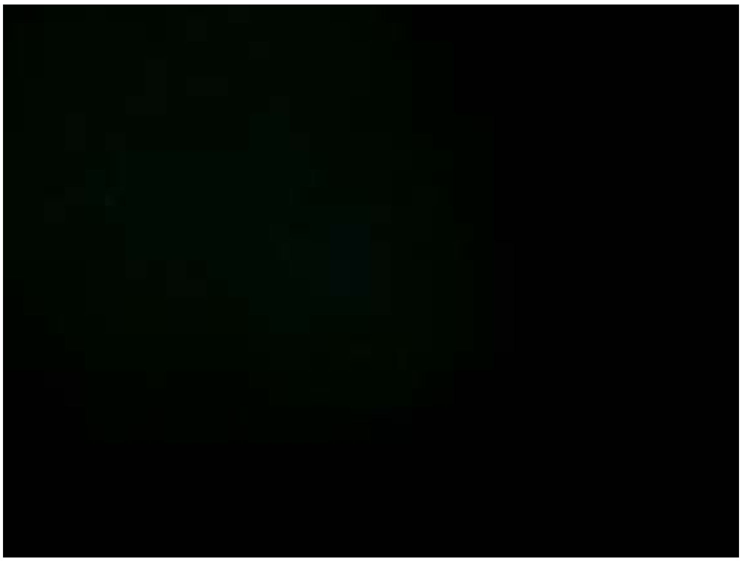
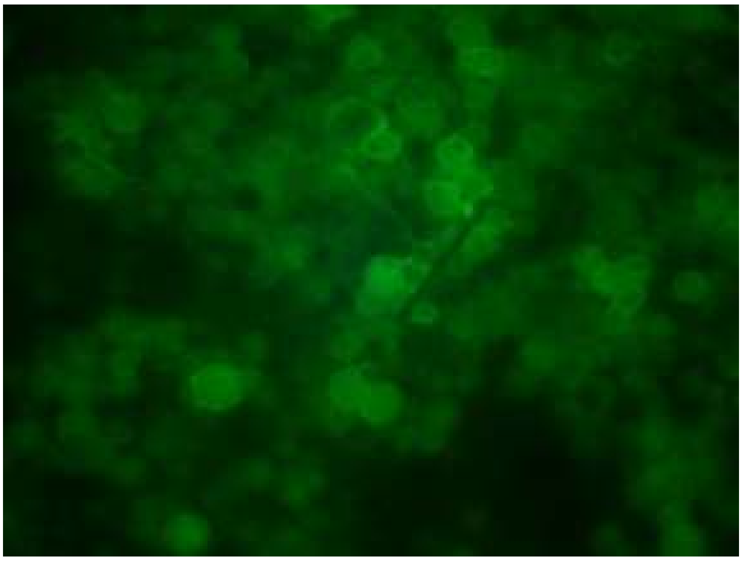
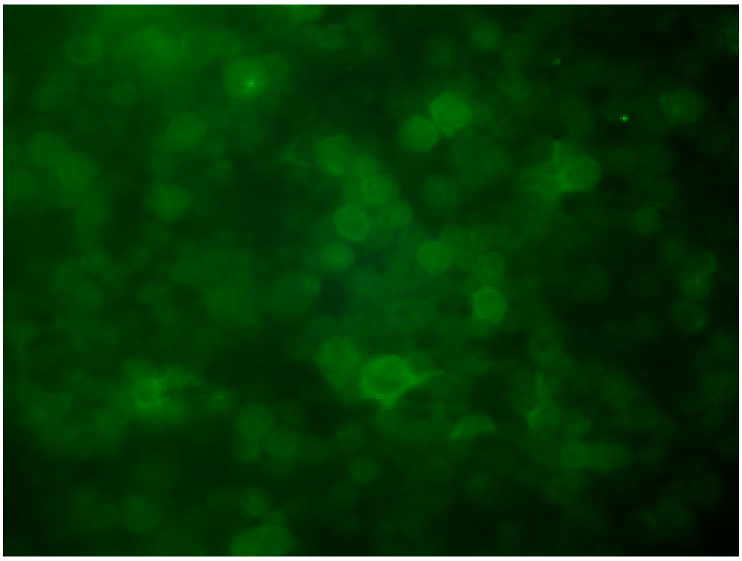


Figure 12: Hepatocyte functional markers- Immunocytochemistry with antibody against human albumin:

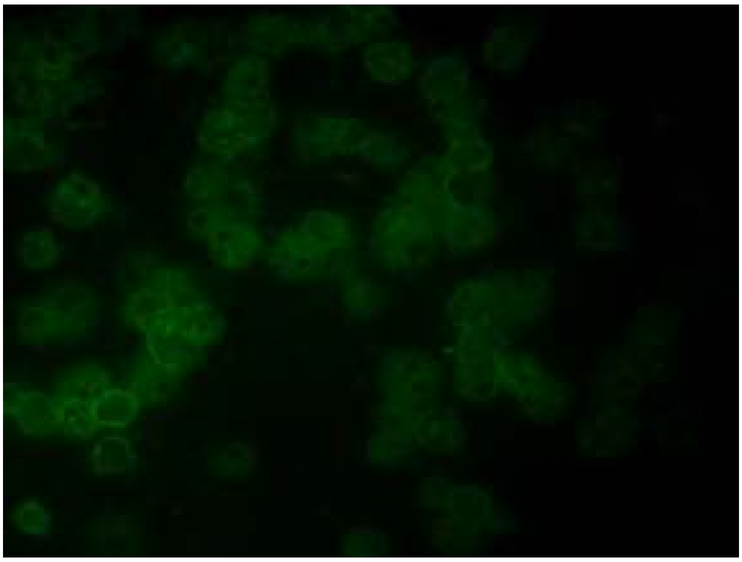
12 a) Cultured cells from patients with cirrhosis and HCC: cytoplasmic staining was noted with this antibody



12 b) Cells from patients with cirrhosis and without HCC: Diffuse staining of the cytoplasm



12 c) Positive control- Human hepatocytes



12d) Negative control- Murine pancreatic beta cell line (Min)

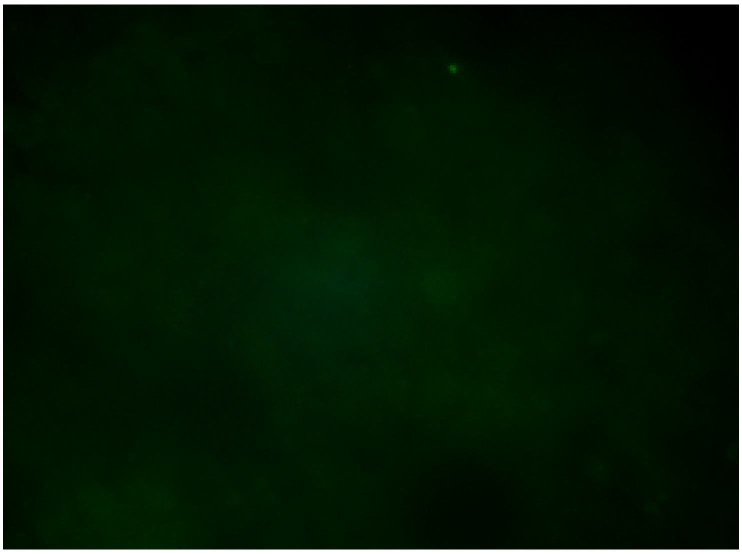
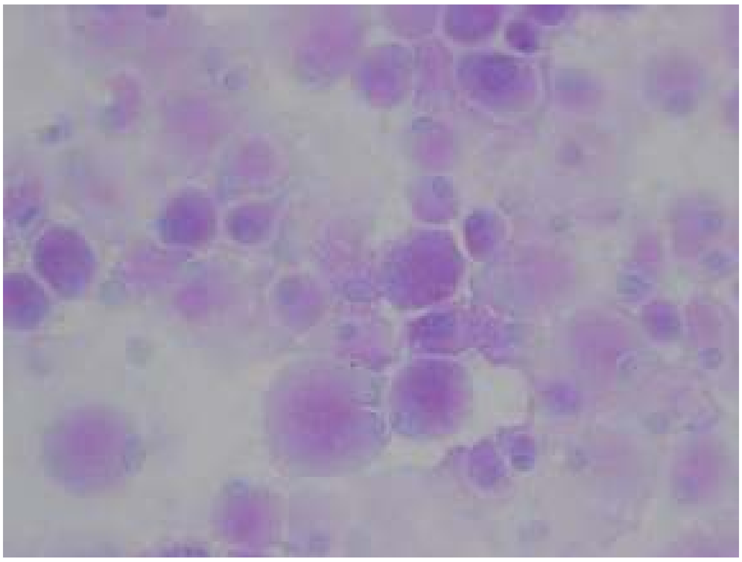
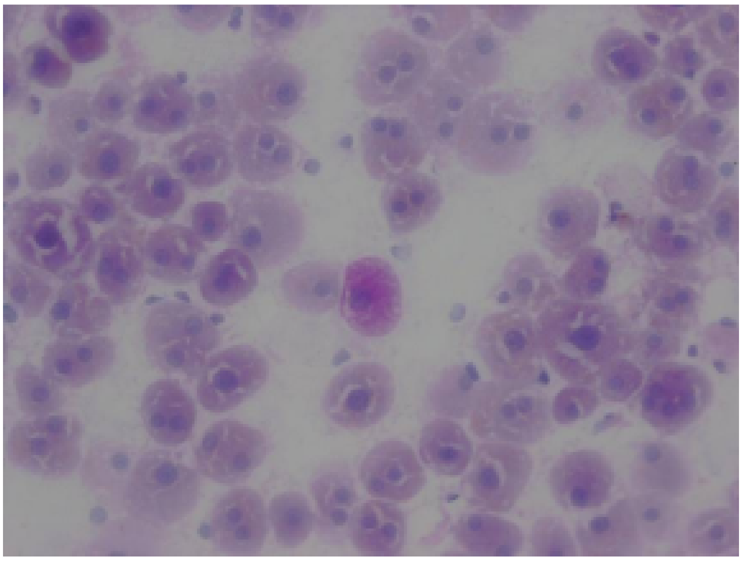


Figure 13: Hepatocyte functional marker- PAS staining for glycogen:

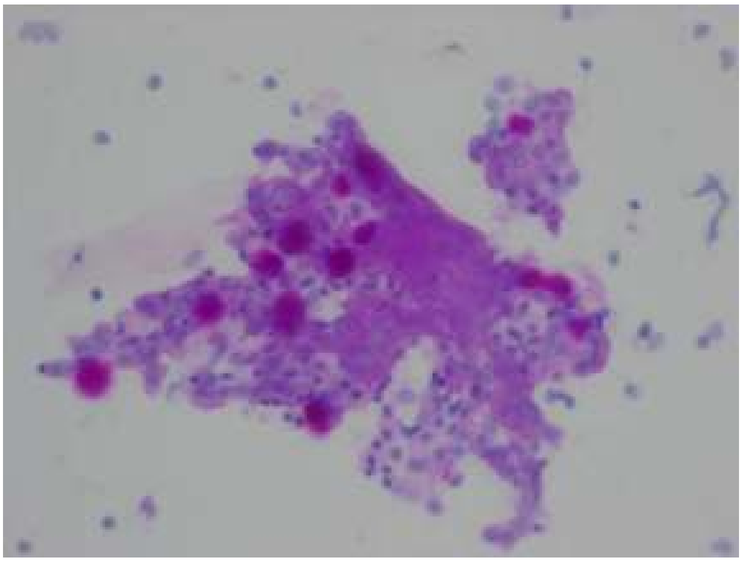
13 a) *In vitro* culture cells from patients with cirrhosis and HCC: characteristic diffuse cytoplasmic staining of the glycogen reserves



13 b) Cultured cells from patients with cirrhosis but without HCC: cytoplasmic staining was similar to the pattern noted in the cells cultured from patients with cirrhosis and HCC



13 c) Positive control- Fine needle aspiration cytology of intestinal smooth muscle cells



13 d) Negative control- Colon cancer cell line (Caco2)

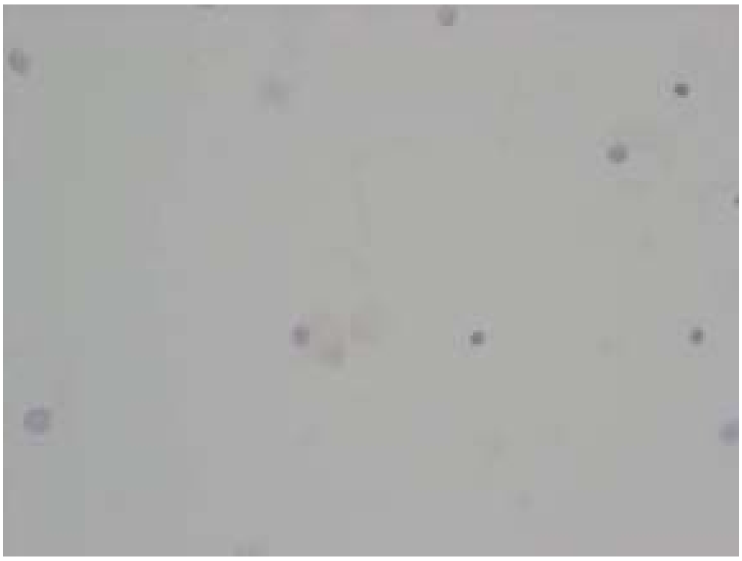
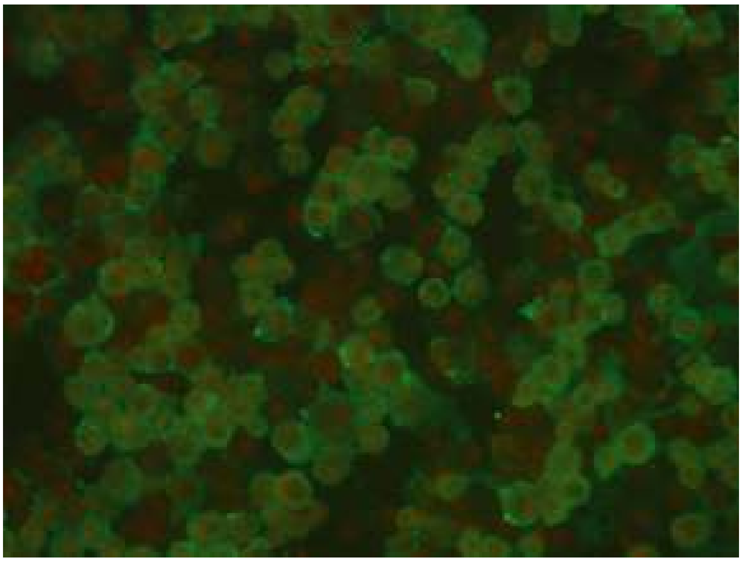
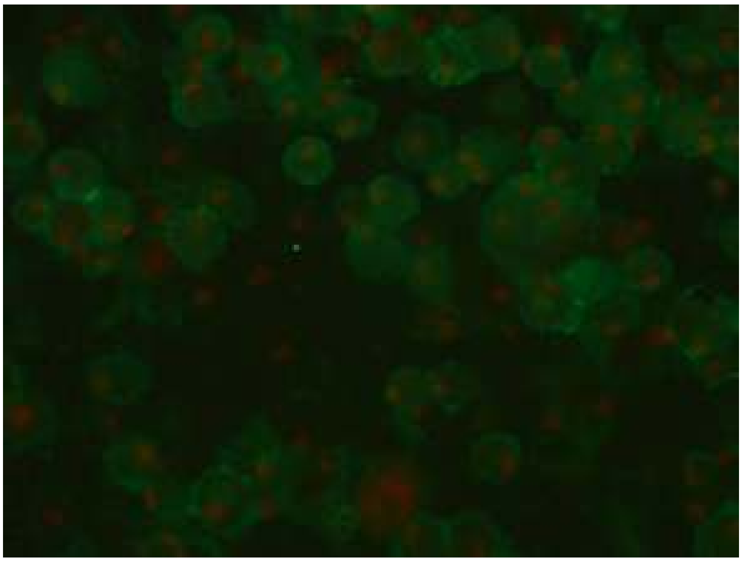


Figure 14: Immunocytochemistry using stem cell marker CD34:

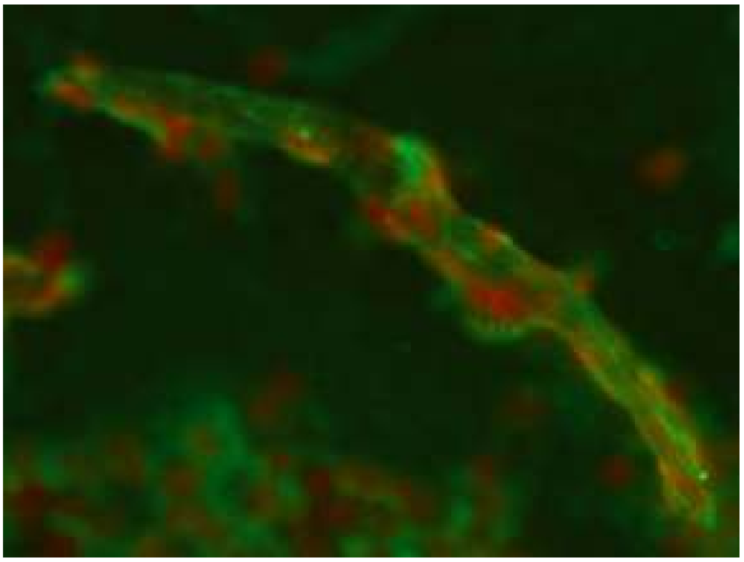
14 a) *In vitro* culture cells from patients with cirrhosis and HCC: Typical membrane staining with CD34



14b) Culture cells from patients with cirrhosis but no HCC: positive membrane staining



14 c) Positive control- Liver endothelial cells (nuclei counterstained with Propidium iodide- red stain)



14 d) Negative control- Murine pancreatic beta cell line (Min Cells)

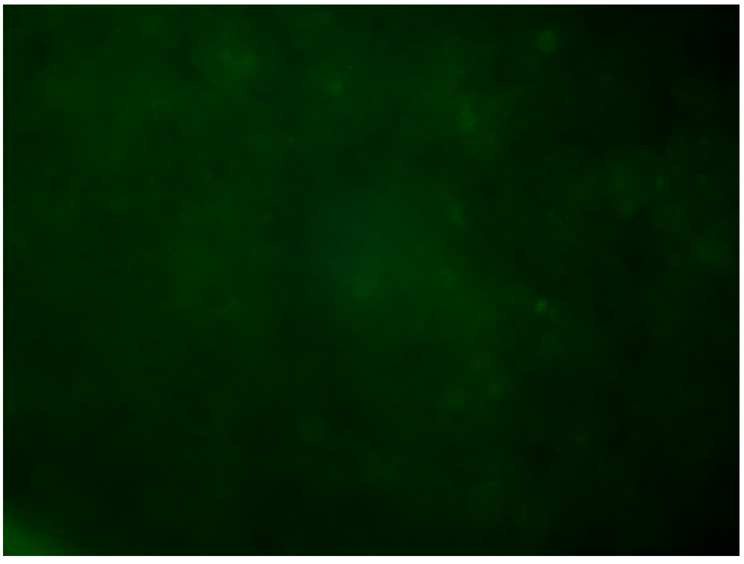
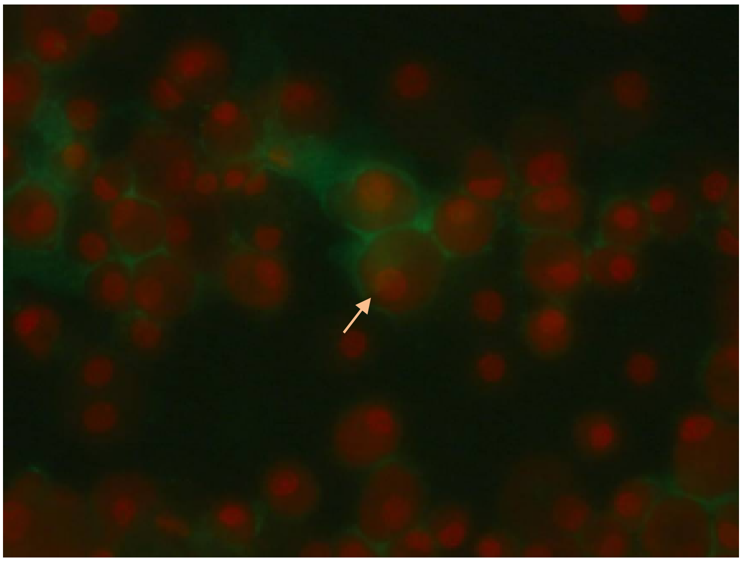
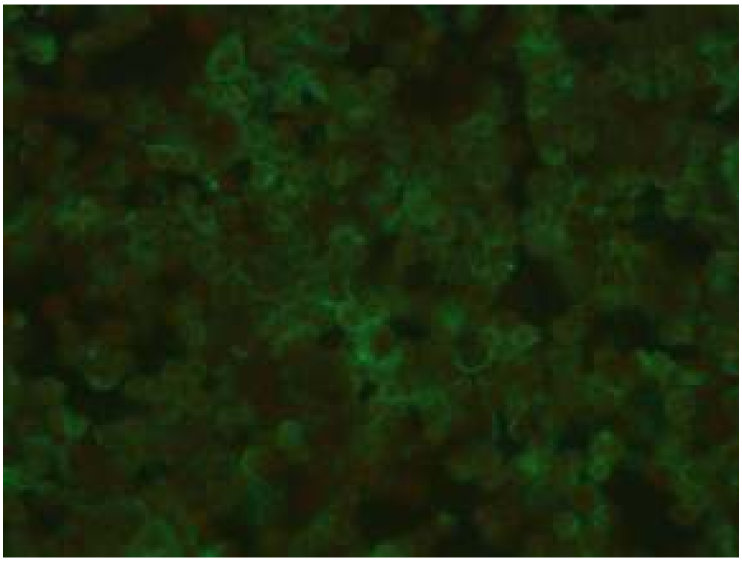


Figure 15: Immunocytochemistry using stem cell marker CD133:

15 a) *In vitro* culture cells from patients with cirrhosis and HCC: limited membrane staining (arrow) was noted with CD133. The nuclei were counterstained with Propidium iodide (red).



15 b) Positive control- Human colon cancer cell line (Caco2)



15 c) Negative control- Murine pancreatic beta cell line (Min Cells)

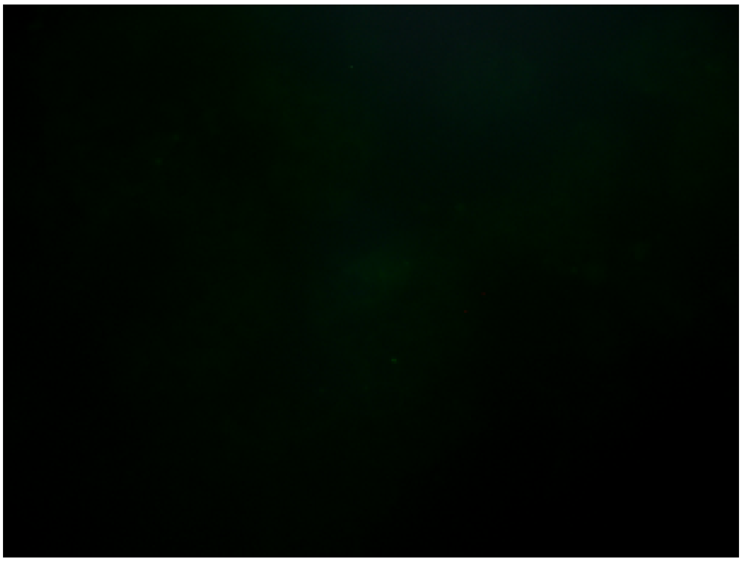
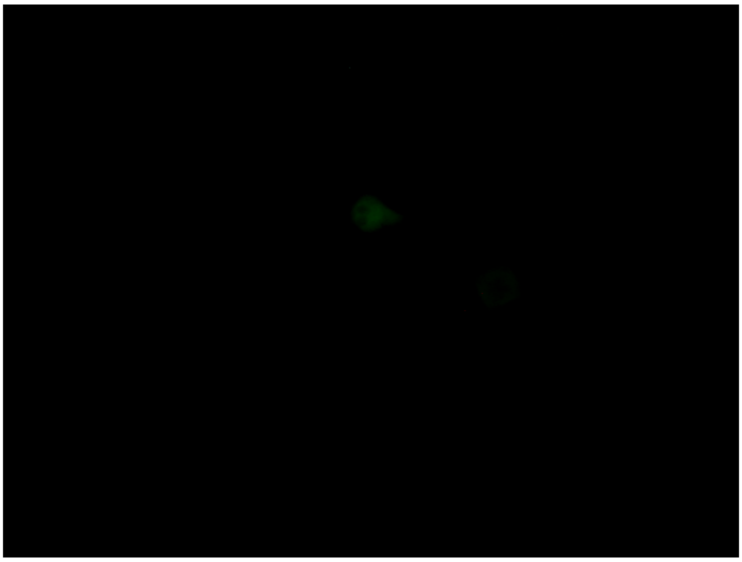
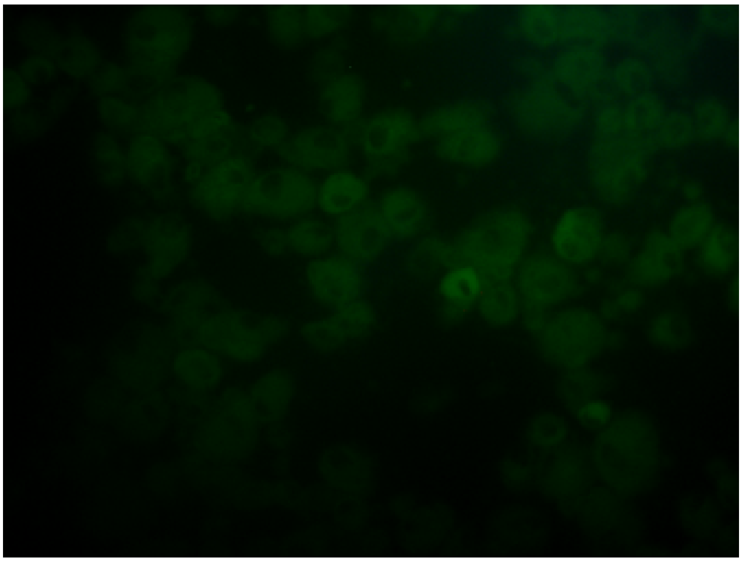


Figure 16: Immunocytochemistry with stem cell marker CD90:

16 a) *In vitro* culture cells from patients with cirrhosis and HCC: Cytoplasmic staining was noted in a very small population of cells



16 b) Positive control- Bone marrow stem cells



16 c) Negative control- Mononuclear cells from peripheral blood

3.4 Discussion:

Curative treatments can only be offered to a minority of HCC patients who present early but tumour recurrence still remains a considerable risk. Unlike LT procedures, there are no strict criteria in the selection of patients for a surgical resection The Milan criteria is widely accepted to allocate cadaveric livers for patients with HCC. A 5 year survival in excess of 70% has been achieved internationally using this selection criteria. However studies aimed at validation of the Milan criteria also identified excellent outcomes in patients outside this strict criteria that lead to the introduction of an extended UCSF criteria.

The pre-operative tumour data such as size and number have been the only basis for all of these selection criteria. The analysis of outcomes using these tumour parameters by different groups produced variable results, also a major drawback was the inclusion tumour variables from post-operative histology (Toso 2008, 2009, Mazzaferro 2009).

The strong predictors of tumour recurrence such as vascular invasion or tumour differentiation are not routinely available pre-operatively. Tumour size has been shown to be as a surrogate marker for vascular invasion, it is the only available variable from radiological imaging to stage HCC. Radiological estimation of tumour morphology is very subjective even with the use of modern imaging modalities. A retrospective study comparing pre-operative radiological staging and post-operative pathological staging in 789 liver transplant recipients concluded that the overall accuracy of pre-operative radiological staging is only 50% (Freeman 2006).

There is a clinical need to develop methods that could the assess tumour biology with the help of simple and minimally invasive diagnostic modalities. The inclusion of such potential markers will further improve our selection criteria and improve the treatment outcomes.

Disseminated tumour cells have been isolated from simple peripheral blood sample specimens in several epithelial cancers. They were linked with early tumour recurrence and poor prognosis. If DTC represent cells detached from primary tumours, their isolation and analysis could shed light on the biological behaviour of cancers.

Studies on patterns of tumour recurrence suggested that the commonest site of recurrence for HCC is the remnant after resection or transplanted liver graft (Ou 2005). The patterns of tumour recurrence were divided into early and late; tumour biology is associated with early recurrence while a de novo carcinogenic process is noted in late recurrence. Nearly 90% of early recurrence is intra-hepatic and such a pattern especially after LT suggests that circulating tumour cells with a potential to migrate and engraft in the liver are undetected during staging investigations. Clinical correlation suggests that the presence of micrometastases in HCC patients is associated with tumour recurrence (Ijichi 2002, Marubashi 2007). It has been possible to detect disseminated tumour cells at a single cell level in bone marrow (BM) and peripheral blood using immunocytochemical and molecular assays. The detection rate of cells using these techniques is in the order of 1-2 cells per million of bone marrow cells. However, the baseline assumption that circulating mRNA is equivalent to circulating cells, the lack of reliability of mRNA detection techniques and the lack of power to accurately predict recurrence has restricted their use clinical practise.

There is some evidence to suggest micrometastases can remain dormant for a prolonged period of time and may or may not develop into clinical metastases (Braun & Pantel 1999). It has been hypothesised that the tumour cells detected in patients who develop recurrence may be phenotypically different from those detected in patients who do not develop tumour recurrence. Sutcliffe et al (2005) demonstrated that despite the presence of DTC, not all patients with HCC developed recurrence (Sutcliffe 2005).

The ability to consistently identify micrometastases and understand their biology at a molecular level (by developing an *in vitro* culture protocol to increase the number of these rare cells) would advance our understanding of HCC and may have an impact on clinical outcome. Experiences from *in vitro* culture of micrometastatic cells from oesophageal cancer (O'Sullivan 1999) suggest that isolation of DTC from foregut cancers is feasible.

The results of our study show that *in vitro* culture of DTC from patients with HCC is possible and that these cells possess characteristic morphological, phonotypic and functional features of hepatocytes.

Of note, however small colonies of hepatocyte-like cells were also isolated in 5 out of 50 patients with cirrhosis (group 2) who did not have HCC, but not from healthy volunteers. The presence of these cells could be explained by the cellular mechanisms behind liver regeneration. It is now well accepted that two physiological forms of liver regeneration occur in response to the type of liver injury. Hepatocytes are the first line cells that help in liver regeneration while the liver progenitor cells serve as second line of defence when hepatocytes can no longer regenerate.

Swanson et al. with the use of genetically modified mice (hereditary tyrosinemia type I) showed that when hepatocytes are not capable of dividing rapidly enough to restore damaged liver, liver progenitor cells and possibly, extra hepatic progenitor cells including those in the bone marrow are recruited to help in the regeneration process.

The cellular response to regeneration is purely determined by the nature of liver injury. Repeated chronic injury as seen in viral hepatitis or steatohepatitis could potentially activate progenitor cells from an extra-hepatic source to differentiate into hepatocytes. The proportion of bone marrow (BM)-derived hepatocytes in human liver have ranged from non-existent in some studies to over 40% and this variation is probably due to differences in the severity of liver parenchymal damage (Alison 2005). The contribution of haematopoietic stem cell (HSC) to liver regeneration in animal experiments and humans remains controversial with data both supporting and challenging the findings. Rodent experiments using a fumarylacetoacetate hydrolase (FAH)-deficient mouse model (fatal hereditary tyrosinemia type I) demonstrated that HSC can differentiate into functional hepatocytes (Lagasse). However, attempts to define their role in restoring a functional liver in human beings are still at an experimental stage (Houlihan 2008).

The detection of circulating hepatocyte-like cells in small group of patients with end-stage liver disease in our study supports a possible extra-hepatic source that helps in liver regeneration. The positive staining with CD34 antibodies suggests a haematopoietic origin of these cells. It was also observed that 60% of patients in this small sub-group had alcohol induced liver injury and a high MELD score, which could indicate a possible relationship between degree of hepatocyte damage and circulating hepatocytes.

The molecular pathways involved in migration of HSC have been investigated in murine and human subjects. It was shown that the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, interactions participate in the mobilization and migration of human HSCs to the liver during injury (Dalakas 2005). Factors regulating long-term engraftment and differentiation of HSCs into hepatocytes are yet to be defined.

The positive immunocytochemistry using stem cell markers (CD34, CD133 and CD90) raises the possibility of the presence of cancer stem cells within cultures derived from our experimental samples (group 1- HCC patients). There is substantial evidence supporting the presence of cancer stem cells (CSC) in many solid organ malignancies, these cells exhibit properties of both normal stem cells and cancer cells and are associated with aggressive cancer behaviour (Visvader 2008).

According to CSC hypothesis, tumours are organized into a hierarchy of heterogeneous cell populations, and only a small subset of cells, termed CSCs or tumour-initiating cells, have the ability to drive and sustain tumour growth. The cancer stem cell hypothesis provides a new insight into hepatocarcinogenesis. Evidence from animal experiments demonstrated that only a minority of HCC are derived from mature hepatocytes. The hypothesis that a human HCC could have a stem cell origin is based on the following indirect evidence ; 1) in a rodent hepatocarcinogenesis model hepatic progenitor cell (HPC)/oval cells have been shown to be the cells of origin of HCC, 2) a considerable proportion of HCCs express hepatocyte progenitor markers that are not generally expressed by mature hepatocytes and 3) many tumours contain a mixture of hepatocellular and cholangiocarcinomatous tumour cells, implying pluri-potent origin (Papoulas 2009).

The role of CSCs in the origin and propagation of HCC is less clear as their expression is not ubiquitous. Several HCC stem cell markers were highlighted in the published studies but their exact roles are still under investigation (Tong 2011). The first recognised and commonly used marker to identify CSC in HCC is CD133, this marker is only confined to a small sub-population of tumour tissue and absent in normal liver. Studies using human HCC cell lines demonstrated that CD133 +ve sub-population of cells had an enhanced clonogenicity in vitro and tumorigenicity in vivo. They also were noted to have properties of stem cells such as ability to self-renew and differentiate into non-hepatocyte lineages (Chan 2007, Song 2008, Tang 2010).

The second widely used CD surface protein for the identification of liver CSCs is CD90. Yang and colleagues found a significant positive correlation of CD90 expression with tumorigenicity and metastatic potential in the panel of liver cell lines tested. It was also reported in their study that CD 90+ve cells were present in peripheral blood samples of patients with aggressive HCC phenotype but not in normal controls or patients with cirrhosis (Yang 2008).

In our study the expression of CD133 and CD90 by cells cultured from patients with HCC was the only discriminating feature compared to those isolated from patients with cirrhosis and no HCC. These markers are only expressed by very small sub-population of HCC and the very low expression by our experimental cells also indicates that the CSC phenotype was probably not retained by the majority of differentiated hepatocytes. This hypothesis was supported by evidence from a mouse model of intestinal cancer where despite all neoplastic cells arising from CD133+ stem cells, only a small fraction of the tumour cells retained CD133 expression (Visuvader 2011)

The origin of CSCs in HCC is controversial with some studies supporting a bone marrow origin while others suggest a liver stem cell origin (Sell 2008). The immunocytochemistry results in this chapter suggests that circulating cells isolated from patients with HCC, express markers (CD34,CD133,CD90) that could associate them with a hematopoietic or stem cell origin.

The immunocytochemistry analysis has shown that the cultured cells had acquired features of hepatocytes. Apart from the low expression of CSC markers there were no features that distinguished cells derived from patients with HCC from those cultured from cirrhosis controls. The malignant potential of DTC cannot be determined accurately by immunocytochemistry alone, and further work is required to confirm whether they originate within bone marrow or represent circulating liver-derived cells.

3.5 Conclusion: The results obtained showed that circulating hepatocyte like cells are present in patients with HCC and chronic liver disease. The true biological behaviour of these cells is not clear.

The aim of the next chapter is to evaluate the malignant nature and the origin of cells cultured from patients with HCC by using molecular assays.

CHAPTER 4: BIOLOGICAL BEHAVIOUR AND ORIGIN OF DISSEMINATED TUMOUR CELLS IN HCC

4.1 Introduction: Disseminated tumour cells have been detected in the bone marrow or peripheral blood of patients with epithelial cancers using immunocytochemistry or molecular assays. However there is only a limited knowledge about the genetic, phenotypic and biological characteristics of these cells. Pantel et al extensively investigated the clinical relevance of the detection of DTC in breast cancer. The largest multicentric study including 4703 breast cancer patients showed that micrometastasis could be detected in 30% of patients and their presence is an independent risk factor for poor overall survival. The results also concluded that despite the associated adverse prognosis, nearly half of the patients identified with DTC did not develop clinical metastases after 10 year follow-up (Pantel 2009).

In our previous study that aimed at identifying an effective diagnostic tool to detect micrometastases in HCC, we analysed bone marrow aspirates from 32 subjects (18-HCC; 9-patients with cirrhosis and 5- healthy volunteers without liver disease). Seventeen (94%) patients with HCC were within the Milan criteria pre-operatively. Explant histology reported microvascular invasion in 8(44%) and poor differentiation in 4(22%). At a median follow-up of 43months it was observed that nearly half of the patients with detectable micrometastases did not develop tumour recurrence (Sutcliffe 2005).

The phenomenon of dormancy has been observed in DTC isolated from patients with breast cancer. Meng et al demonstrated that DTC can be detected in patients with no evidence of clinical metastases up to 22 years after curative mastectomy. The underlying molecular mechanisms that can maintain DTC dormancy or activate them to form clinical metastases have yet to be identified. It is important to further characterise DTC and to understand the homeostatic mechanisms such as anti-tumour immune response or angiogenesis suppression to develop potential therapeutic agents.

Our previous experiment demonstrated that DTC can be isolated in patients with HCC and that they have morphological features and a functional phenotype similar to hepatocytes (Immunocytochemistry analysis using relevant antibodies- Chapter 3). Interestingly similar cells were also detected in a small proportion of patients with chronic liver disease but with no clinical evidence of HCC. The H&E staining and immunocytochemistry assessment could not demonstrate any strong differences between these two groups of cells, particularly the malignant potential of cultured cells from patients with HCC.

Liver cirrhosis is the strongest risk factor for HCC, distinguishing pre-neoplastic lesions from HCC is still a challenge even for expert histopathologist. Pathological examination is considered as gold standard to discriminate HCC from cirrhotic nodules. Although immunostaining plays a very important role in the clinical diagnosis of HCC, it has diagnostic limitations. (Saad 2004, Llovet 2006). Genomic-wide cDNA microarrays and quantitative real-time reverse transcription-polymerase assay studies have been attempted to develop a molecular index which is efficient at categorizing liver tissue samples as normal or cirrhotic liver or HCC, however, none of them were able to produce a signature that is clinically acceptable (Paradis 2003, Okabe 2001, Xiao 2001, Shirota 2001)

Llovet et al have developed a 3-gene set that allows a reliable diagnosis of HCC and can also discriminate between HCC and dysplasia or cirrhosis. This 3 gene signature consisting of

LYVE1, glypican-3 and survivin is shown to have a sensitivity of 95%, specificity of 94% and an accuracy of 94% in discriminating HCC from dysplastic or cirrhotic nodules. The 3-gene set was also validated in 29 samples (Llovet 2006). We selected this 3 gene signature along with TGF-alpha and p53 to discriminate DTC from cells isolated from patients with cirrhosis (Yeh 2007, Anzola 2004, Zhang 2004). The cells cultured from patients with HCC were further assessed for the expression of a wide range of malignant genes shown to be associated with poor prognosis in solid organ tumours and HCC.

The results of our previous experiment also demonstrated the expression of bone marrow stem cell (BMSC) phenotype by the cultured cells from patients with HCC which raised the possible BM origin of DTC. Bone marrow is a rich source for pleuripotent stem cells and has been proposed as the source for cancer stem cells in several epithelial cancers. The Helicobacter pylori gastric cancer mouse model showed that BMSC are inherently mutagenic and can transform into malignant cells with an appropriate stimulus (Houghton 2005).

The evidence for BM involvement in hepatocarcinogenesis is still controversial (Theise 2000, Lagasse 2000, Kubota 2008, Sell 2009). In-view of considerable evidence both supporting and disputing it, we attempted to investigate the association between BMSC and our cultured cells by comparing their gene expression profiles.

The aim of this chapter is therefore: 1) to investigate the *in vitro* neoplastic behaviour of cultured cells from patients with HCC 2) to determine the origin of DTC.

4.2 Materials and methods:

In an attempt to differentiate cells cultured from patients with cirrhosis and HCC and a control group with cirrhosis and no identifiable HCC, we used the 3 gene signature described by Llovet et al and the expression of TGF-alpha and p53. None of the cirrhosis control group has developed HCC at the latest follow up.

The 3- gene expressions were evaluated by using qPCR. Single-step real-time qPCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems Inc, CA, USA). qPCR was carried out in 20-µl reaction mixtures using the TaqMan gene expression master mix (Applied Biosystems Inc, CA, USA) in 96 well plates using the protocol described in section 2.9.5. Levels of glypican 3, LYVE1 and survivin were assessed and an external standard template containing human GAPDH cDNA was included in each run to generate a standard curve. qPCR probes were purchased from Applied Biosystems Inc.

TGF-alpha and p53 analysis was performed by RT-PCR using a PCR selection kit (Invitrogen, UK), following the company protocol. Primers were purchased from Eurofins MWG Operon (Germany), apart from GAPDH which was designed in-house.

Primer sequences:

<u>Glypican-3</u>

Forward**:**	CTGACTGACCGCGTTACTCCCACA
Reverse:	TAGCAGCATCGCCACCAGCAAGCA

<u>Survivin:</u>

Forward:	TAGGATCCATGGGTGCCCCGACG
Reverse:	ACCTCGAGCTCAATCCATGGCAGCC

<u>TGFα</u>

Forward:	CCGTAAAATGGTCCCCT
Reverse:	CTCTTCAGACCACTGTTT

<u>p53</u>

Forward:	CACTGCCATGGAGGAG
Reverse:	GTCAGTCTGAGTCAGG

<u>GAPDH</u>

Forward;	CCATGGGGAAGGTGAA
Reverse:	TCTTACTCCTTGGAGGC

Description of selected genes:

Glypican 3 (GPC3): Is a member of the glypican family of cell-surface heparan sulfate proteoglycans and serves as a co-receptor for heparin-binding growth factors. It is reported that GPC3 messenger RNA (mRNA) levels are over expressed in 75% of HCCs but not in focal nodular hyperplasia or cirrhosis (Zhu 2001). Hsu et al demonstrated that GPC3 mRNA was detectable in 74.8% of primary and recurrent HCCs but only 3.2% of normal livers. Cappuro et al suggested that GPC3 promotes the growth of HCC by stimulating the canonical Wnt pathway (Cappuro 2005). Although GPC3 mRNA expression was noted in foetal livers and placenta suggesting its expression by regenerating hepatocytes, Nakatsura et al showed no such correlation in their study (Nakatsura 2003).

Survivin: Is an oncofoetal protein that belongs to the inhibitor of apoptosis proteins family. It is undetectable in terminally differentiated adult tissues but widely expressed by foetal tissue and a variety of cancer cells. Survivin inhibits the activation of caspases leading to negative regulation of apoptosis. It has been implicated in the carcinogenesis of neuroblastoma, colorectal, non–small-cell lung and gastric cancer. Survivin expression was increased (3.3- fold) in early and (24-fold) in advanced HCC, compared with normal tissue (Llovet 2006, Montorsi 2007). Studies investigating the expression and function of survivin using human HCC cell lines observed that its expression correlates with cell proliferation and tumour progression (Ito 2000).

Lymphatic vessel endothelial hyaluronan receptor (LYVE)-1: Is a transmembrane glycoprotein that is expressed by normal lymphatic endothelium and sinusoidal cells in the liver.

Although the physiological role of this receptor is not clearly defined, it has been associated with tumour metastases and poor prognosis. LYVE-1 is reportedly down-regulated in solid tumours, such as breast, lung, and endometrial cancer suggesting destruction of the lymphatics by tumour invasion. LYVE-1 is also down regulated in HCC (Carreira 2001), Llovet et al observed a 1.6-fold decreased in dysplastic nodules and a 20-fold decreased in early HCC.

Transforming growth factor alpha (TGF-α): Growth factor signal pathways regulate cell proliferation, differentiation, and apoptosis and aberrant or over expression of these factors could lead to neoplasia. TGF-α is a mitogen synthesized as a transmembrane polypeptide and has been implicated in the molecular pathogenesis of HCC. Transgenic mouse models showed that over expression of TGF-α resulted in increased hepatocyte proliferation, dysplasia, adenoma and HCC (Jo 1999, Yeh 2007). Chug et al showed that TGF-alpha mRNA expression was significantly elevated in patients with HCC when compared with chronic viral hepatitis controls (Chung 2000).

Protein 53(p53): Is a nuclear phosphoprotein coded by a tumour suppressor gene p53. It functions as a transcriptional regulatory protein and has a critical role in the regulation of the cell cycle. Tumour suppressor genes are of great importance in human carcinogenesis and mutations in the p53 gene have been reported in a variety of human cancers including HCC. Studies investigating the incidence of mutations or over expression of p53 in different geographic HCC populations have yielded variable results. The incidence of p53 mutations in HCC throughout the world varies between 15% in Europe and 42% in China, with a worldwide frequency of 27% (Bressac 1990, Ozturk 1991, An 2001). A high level of cellular p53 protein due to overexpression of p53 gene correlates with the degree of malignancy (Guan 2006). Abnormal expression of p53 in HCC is regarded as an indicator of the genetic instability and biological aggressiveness of the tumour (Anzola 2003).

The expression of cancer-associated genes in cells cultured from patients with HCC was studied by microarray analysis. More than 300 microarray studies have investigated deregulated genes in HCC to-date, however, the challenge of identifying clinically relevant genes persists (Andrisani 2011). Following an extensive literature search we considered those genes that have a potential role in the diagnosis, pathogenesis and predicting the prognosis of HCC as bench mark to analyse the cultured cells from patients with HCC. A list of genes that are expressed by HCC as well as those associated with poor prognosis both in HCC and other solid tumours are displayed in Tables 1, 2 and 3 along with appropriate references.

As the cells isolated from patients with cirrhosis were limited by number it was not possible to include them in this analysis. The gene expression profiles of DTC were compared with controls that consisted of primary human hepatocytes and purified CD34^{+ve} bone marrow stem cells.

Table 1: 10 genes previously described as diagnostic markers of HCC

Ref	Gene Symbol	Gene Name	Reference
1	KRT19	Cytokeratin 19	Grabarević, Z. *et al*. Comparative analysis of hepatocellular carcinoma in men and dogs. Coll. Antropol. 33, 811-814 (2009).
2	TM4SF1	Transmembrane 4 L six family member 1	Dong, H. *et al*. Gene expression profile analysis of human hepatocellular carcinoma using SAGE and LongSAGE. BMC Med. Genom. 2, 5 (2009).
3	AFP	Alpha fetoprotein	Johnson, P.J. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. Clin. Liver Dis. 5, 145–59 (2001).
4	GPC3	Glypican 3	Hsu, H.C., Cheng, W. & Lai, P.L. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biologic significance and temporospatial distribution. Cancer Res. 57, 5179–5184 (1997).
5	PEG10	Paternally expressed 10	Tsou, A.P. *et al*. Over expression of a novel imprinted gene, PEG10, in human hepatocellular carcinoma and in regenerating mouse livers. J. Biomed. Sci. 10, 625-635 (2003).
6	CPE	Carboxypeptidase E	Wang, W., Peng, J.X., Yang, J.Q., Yang, L.Y. Identification of Gene Expression Profiling in Hepatocellular Carcinoma Using cDNA Microarrays. Dig. Dis. Sci. 54, 2729–2735 (2009).
7	DKK1	Dickkopf homolog 1	Patil, M.A. *et al*. An integrated data analysis approach to characterize genes highly expressed in hepatocellular carcinoma. Oncogene 24, 3737-3747 (2005).
8	NEDD4L	Neural precursor cell expressed, developmentally down-regulated 4-like	Lee, H.S. *et al*. Novel candidate targets of Wnt/β-catenin signalling in hepatoma cells. Life Sci. 80, 690–698 (2007).
9	NEK3	NIMA (never in mitosis gene a)-related kinase 3	Hernández, M., Almeida, T.A. Is there any association between nek3 and cancers with frequent 13q14 deletion? Cancer Invest. 24, 682-688 (2006).
10	FDPS	Farnesyl diphosphate synthase	Sung, Y.K. *et al*. Glypican-3 is over expressed in human hepatocellular carcinoma. Cancer Sci. 94, 259-262 (2003).

Table 2: List of 18 genes previously described to be associated with poor prognosis in HCC

Ref	Gene symbol	Gene Name	Reference
1.	IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	Jeng, Y.M. *et al*. RNA-binding protein insulin-like growth factor II mRNA-binding protein 3 expression promotes tumour invasion and predicts early recurrence and poor prognosis in hepatocellular carcinoma. Hepatol. 48, 1118-1127 (2008).
2	RAB23	Member RAS oncogene family	Liu, Y.J. *et al*. Rab23 is a potential biological target for treating hepatocellular carcinoma. World J. Gastroenterol. 13, 1010-1017 (2007).
3	NQO1	NAD(P)H dehydrogenase, quinone 1	Chiu, M.M., Ko, Y.J., Tsou, A.P., Chau, G.Y., Chau, Y.P. Analysis of NQO1 polymorphisms and p53 protein expression in patients with hepatocellular carcinoma. Histol. Histopathol. 24, 1223-1232 (2009).
4	ROCK2	Rho-associated coiled-coil containing protein kinase 2	Wong, C.C., Wong, C.M., Tung, E.K., Man, K., Ng, I.O. Rho-kinase 2 is frequently over expressed in hepatocellular carcinoma and involved in tumour invasion. Hepatol. 49, 1583-1594 (2009).
5	DLK1	Delta-like 1 homolog (Drosophila)	Jin, Z.H., Yang, R.J., Dong, B., Xing, B.C. Progenitor gene DLK1 might be an independent prognostic factor of liver cancer. Expert Opin. Biol. Ther. 8, 371-377 (2008).
6	ZHX2	Zinc fingers and homeoboxes 2	Hu, S. *et al*. Expression of zinc-fingers and homeoboxes 2 in hepatocellular carcinogenesis: a tissue microarray and clinicopathological analysis. Neoplasma 54, 207-211 (2007).
7	LIN28B	Lin-28 homolog B (C. elegans)	Viswanathan, S.R. *et al*. Lin28 promotes transformation and is associated with advanced human malignancies. Nat. Genet. 41, 843-848 (2009).
8	KRT19	Cytokeratin 19	Yang, X.R. *et al*. Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. Clin. Cancer Res. 14, 3850-3859 (2008).
9	DKK1	Dickkopf homolog 1	Yu, B. *et al*. Elevated expression of DKK1 is associated with cytoplasmic/nuclear beta-catenin accumulation and poor prognosis in hepatocellular carcinomas. J. Hepatol. 50, 948-957 (2009).
10	PEG10	Paternally expressed 10	Ip, W.K. *et al*. Identification of PEG10 as a progression related biomarker for hepatocellular carcinoma. Cancer Lett. 250, 284-291 (2007).
11	CCR6	Chemokine (C-C motif) receptor 6	Uchida, H. *et al*. Chemokine receptor CCR6 as a prognostic factor after hepatic resection for hepatocellular carcinoma. J. Gastroenterol. Hepatol. 21, 161-168 (2006).

12	BMP4	Bone morphogenetic protein 4	Maegdefrau, U. *et al*. Bone morphogenetic protein 4 is induced in hepatocellular carcinoma by hypoxia and promotes tumour progression. J. Pathol. 218, 520-529(2009)
13	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	Shi, G.M. *et al*. Identification of side population cells in human hepatocellular carcinoma cell lines with stepwise metastatic potentials. J. Cancer Res. Clin. Oncol. 134, 1155-1163 (2008).
14	SPP1	Secreted phosphoprotein 1	Pan, H.W. *et al*. Over expression of osteopontin is associated with intrahepatic metastasis, early recurrence, and poorer prognosis of surgically resected hepatocellular carcinoma. Cancer 98, 119-127 (2003).
15	CCL20	Chemokine (C-C motif) ligand 20	Yamauchi, K., Akbar, S.M., Horiike, N., Michitaka, K., Onji, M. Increased serum levels of macrophage inflammatory protein-3alpha in hepatocellular carcinoma: relationship with clinical factors and prognostic importance during therapy. Int. J. Mol. Med. 11, 601-605 (2003).
16	GSTA4	Glutathione S-transferase alpha 4	McGlynn, K.A. *et al*. Susceptibility to aflatoxin B1-related primary hepatocellular carcinoma in mice and humans. Cancer Res. 63, 4594–4601 (2003).
17	ROBO1	Roundabout, axon guidance receptor homolog 1	Avci, M.E., Konu, O., Yagci, T. Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression. BMC Cancer 8, 392 (2008).
18	CTGF	Connective tissue growth factor	Mazzocca, A. *et al*. Down-regulation of connective tissue growth factor by inhibition of transforming growth factor beta blocks the tumour-stroma cross-talk and tumour progression in hepatocellular carcinoma. Hepatol. 51, 523-534 (2010).

Table 3: List of 43 genes associated with poor outcome in non-HCC solid tumours

Ref	Gene Symbol	Gene name	Reference
1	S100P	S100 calcium binding protein P	Dairkee, S.H. *et al*. Immutable functional attributes of histological grade revealed by context-independent gene expression in primary breast cancer cells. Cancer Res. 69, 7826-7834 (2009).
2	FBLN1	Fibulin 1	Roger, P., Pujol, P., Lucas, A., Baldet, P., Rochefort, H. Increased immunostaining of fibulin-1, an estrogen-regulated protein in the stroma of human ovarian epithelial tumours. Am. J. Pathol. 153, 1579-1588 (1998).
3	CCL20	Chemokine (C-C motif) ligand 20	Chang, K.P. *et al*. Macrophage inflammatory protein-3alpha is a novel serum marker for nasopharyngeal carcinoma detection and prediction of treatment outcomes. Clin. Cancer Res. 14, 6979-6987 (2008).
4	LAMB1	Laminin, beta 1	Bresalier, R.S. *et al*. The laminin alpha 1 chain Ile-Lys-Val-Ala-Val (IKVAV)-containing peptide promotes liver colonization by human colon cancer cells. Cancer Res. 55, 2476-2480 (1995).
5	LGR5	Leucine-rich repeat containing G protein-coupled receptor 5	McClanahan, T. *et al*. Identification of over expression of orphan G protein-coupled receptor GPR49 in human colon and ovarian primary tumours. Cancer Biol. Ther. 5, 419-426 (2006).
6	STC2	Stanniocalcin 2	Meyer, H.A. *et al*. Identification of stanniocalcin 2 as prognostic marker in renal cell carcinoma. Eur. Urol. 55, 669-678 (2009).
7	MUC15	Mucin 15, cell surface associated	Huang, J. *et al*. Over expression of MUC15 activates extracellular signal-regulated kinase 1/2 and promotes the oncogenic potential of human colon cancer cells. Carcinogenesis 30, 1452-1458 (2009).
8	IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1	Köbel, M. *et al*. Expression of the RNA-binding protein IMP1 correlates with poor prognosis in ovarian carcinoma. Oncogene 26, 7584-7589 (2007).
9	ETV4	Ets variant 4	Cheville, J.C. *et al*. Gene panel model predictive of outcome in men at high-risk of systemic progression and death from prostate cancer after radical retropubic prostatectomy. J. Clin. Oncol. 26, 3930-3936 (2008).
10	KIAA1199	KIAA1199	Matsuzaki, S. *et al*. Clinicopathologic significance of KIAA1199 over expression in human gastric cancer. Ann. Surg. Oncol. 16, 2042-2051 (2009).
11	TYRO3	TYRO3 protein tyrosine	Zhu, S. *et al*. A genomic screen identifies TYRO3 as

		kinase	a MITF regulator in melanoma. PNAS 106, 17025-17030 (2009).
12	BMP4	Bone morphogenetic protein 3	Montesano, R., Sarközi, R., Schramek, H. Bone morphogenetic protein-4 strongly potentiates growth factor-induced proliferation of mammary epithelial cells. Biochem. Biophys. Res. Commun. 374, 164-168 (2008).
13	JAG1	Jagged 1	Reedijk, M. *et al*. High-level co expression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res. 65, 8530-8537 (2005).
14	EGLN3	Egl nine homolog 3 (C. elegans)	Couvelard, A. *et al*. Over expression of the oxygen sensors PHD-1, PHD-2, PHD-3, and FIH Is associated with tumour aggressiveness in pancreatic endocrine tumours. Clin. Cancer Res. 14, 6634-6639 (2008).
15	ADORA2B	Adenosine A2b receptor	Li, S., Huang, S., Peng, S.B. Over expression of G protein-coupled receptors in cancer cells: involvement in tumour progression. [Review] Int. J. Oncol. 27, 1329-1339 (2005).
16	GALNT13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide Nacetylgalactosaminyltransferase 13(GalNAc-T13)	Berois, N. *et al*. ppGalNAc-T13: a new molecular marker of bone marrow involvement in neuroblastoma. Clin. Chem.52, 1701-1712 (2006).
17	PGC	Progastricsin (pepsinogen C)	Alvarez, M.L., González, L.O., Barbón, J.J., Astudillo, A., Vizoso, F.J. Expression and clinical significance of pepsinogen C in uveal melanomas. Int. J. Biol. Markers 19, 240-244 (2004).
18	FDPS	Farnesyl diphosphate synthase	Ettinger, S.L. *et al*. Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. Cancer Res. 64, 2212-2221 (2004).
19	PAGE1	Pantigen family, member 1	Chen, M.E., Lin, S.H., Chung, L.W., Sikes, R.A. Isolation and characterization of PAGE-1 and GAGE-7. New genes expressed in the LNCaP prostate cancer progression model that share homology with melanoma-associated antigens. J. Biol. Chem. 273, 17618-17625 (1998).
20	S100A1	S100 calcium binding protein A1	DeRycke, M.S. *et al*. S100A1 expression in ovarian and endometrial endometrioid carcinomas is a prognostic indicator of relapse-free survival. Am. J. Clin. Pathol. 132, 846-856 (2009).
21	CA9	Carbonic anhydrase IX	Korkeila, E. *et al*. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. Br. J. Cancer 100, 874-880 (2009).

22	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	Fu, X.D. *et al*. Extra-nuclear signalling of progesterone receptor to breast cancer cell movement and invasion through the actin cytoskeleton. PLoS One. 3 e2790 (2008).
23	MKKS	McKusick-Kaufman syndrome	Kim, J.C. *et al*. Gene expression profiling: canonical molecular changes and clinicopathological features in sporadic colorectal cancers. World J. Gastroenterol. 14, 6662-6672 (2008).
24	FAM162A	Family with sequence similarity 162, member A	Cho, Y.E., Kim, J.Y., Kim, Y.W., Park, J.H., Lee, S. Expression and prognostic significance of human growth and transformation-dependent protein in gastric carcinoma and gastric adenoma. Human Pathol. 40, 975-981 (2009).
25	AHCY	Adenosylhomocysteinase	Scotto, L. *et al*. Identification of copy number gain and over expressed genes on chromosome arm 20q by an integrative genomic approach in cervical cancer: potential role in progression. Genes Chromosomes Cancer 47, 755-765 (2008).
26	DLK1	Delta-like 1 homolog (Drosophila)	Yin, D. *et al*. DLK1: increased expression in gliomas and associated with oncogenic activities. Oncogene 25, 1852-1861 (2006).
27	PTTG1IP	Pituitary tumor-transforming 1 interacting protein	Stratford, A.L. *et al*. Pituitary tumour transforming gene binding factor: a novel transforming gene in thyroid tumorigenesis. J. Clin. Endocrinol. Metab. 90, 4341-4349 (2005).
28	S100A3	S100 calcium binding protein A3	Liu, J. *et al*. In silico analysis and verification of S100 gene expression in gastric cancer. BMC Cancer 8, 261 (2008).
29	CKMT1A /CKMT1B	Creatine kinase, mitochondrial 1A/1B.	Cimino, D. *et al*. Identification of new genes associated with breast cancer progression by gene expression analysis of predefined sets of neoplastic tissues. Int. J. Cancer 123, 1327-1338 (2008).
30	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	Tsunoda, S. *et al*. ABCG2 expression is an independent unfavourable prognostic factor in oesophageal squamous cell carcinoma. Oncology 71, 251-258 (2006).
31	SPP1	Secreted phosphoprotein 1	Likui, W, Hong, W, Shuwen, Z. Clinical significance of the upregulated osteopontin mRNA expression in human colorectal cancer. J. Gastrointest. Surg. 14, 74-81 (2010).
32	FAM57A	Family with sequence similarity 57, member A	Pan, D., Wei, L., Yao, M., Wan, D., Gu, J. Down-regulation of CT120A by RNA interference suppresses lung cancer cells growth and sensitizes to ultraviolet-induced apoptosis. Cancer Lett. 235, 26-

			33 (2006).
33	DSG2	Desmoglein 2	Kurzen, H., Münzing, I., Hartschuh, W. Expression of desmosomal proteins in squamous cell carcinomas of the skin. J. Cutan. Pathol. 30, 621-630 (2003).
34	ENO2	Enolase 2 (gamma, neuronal)	Yeh, C.S. *et al*. Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers. Oncol. Rep.19, 81-91 (2008).
35	IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	Li, D. *et al*. IMP3 is a novel prognostic marker that correlates with colon cancer progression and pathogenesis. Ann. Surg. Oncol. 16, 3499-3506 (2009).
36	MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	Liang, J.J. *et al*. Expression of MAP4K4 is associated with worse prognosis in patients with stage II pancreatic ductal adenocarcinoma. Clin. Cancer Res. 14, 7043-7049 (2008).
37	SQLE	Squalene epoxidase	Helms, M.W. *et al*. Squalene epoxidase, located on chromosome 8q24.1, is upregulated in 8q+ breast cancer and indicates poor clinical outcome in stage I and II disease. Br. J. Cancer. 99, 774-780 (2008).
38	ROBO1	Roundabout, axon guidance receptor, homolog 1 (Drosophila)	Wang, B. *et al*. Induction of tumour angiogenesis by Slit-Robo signalling and inhibition of cancer growth by blocking Robo activity. Cancer Cell 4, 19-29 (2003).
39	ETV5	Ets variant gene 5	Chotteau-Lelièvre, A. *et al*. Prognostic value of ERM gene expression in human primary breast cancers. Clin. Cancer Res. 10, 7297-7303 (2004).
40	S100A2	S100 calcium binding protein A2	Ohuchida, K. *et al*. Over-expression of S100A2 in pancreatic cancer correlates with progression and poor prognosis. J. Pathol. 213, 275-282 (2007).
41	CTGF	Connective tissue growth factor	Pandey, D.P. *et al*. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. EMBO J. 28, 523-532 (2009).
42	SMYD2	SET and MYND domain containing 2	Komatsu, S. *et al*. Over expression of SMYD2 relates to tumour cell proliferation and malignant outcome of oesophageal squamous cell carcinoma. Carcinogenesis 30, 1139-1146 (2009).
43	RPS6KB1	Ribosomal protein S6 kinase, 70kDa, polypeptide 1	van der Hage, J.A. *et al*. Over expression of P70 S6 kinase protein is associated with increased risk of locoregional recurrence in node-negative premenopausal early breast cancer patients. Br. J. Cancer 90, 1543-1550 (2004).

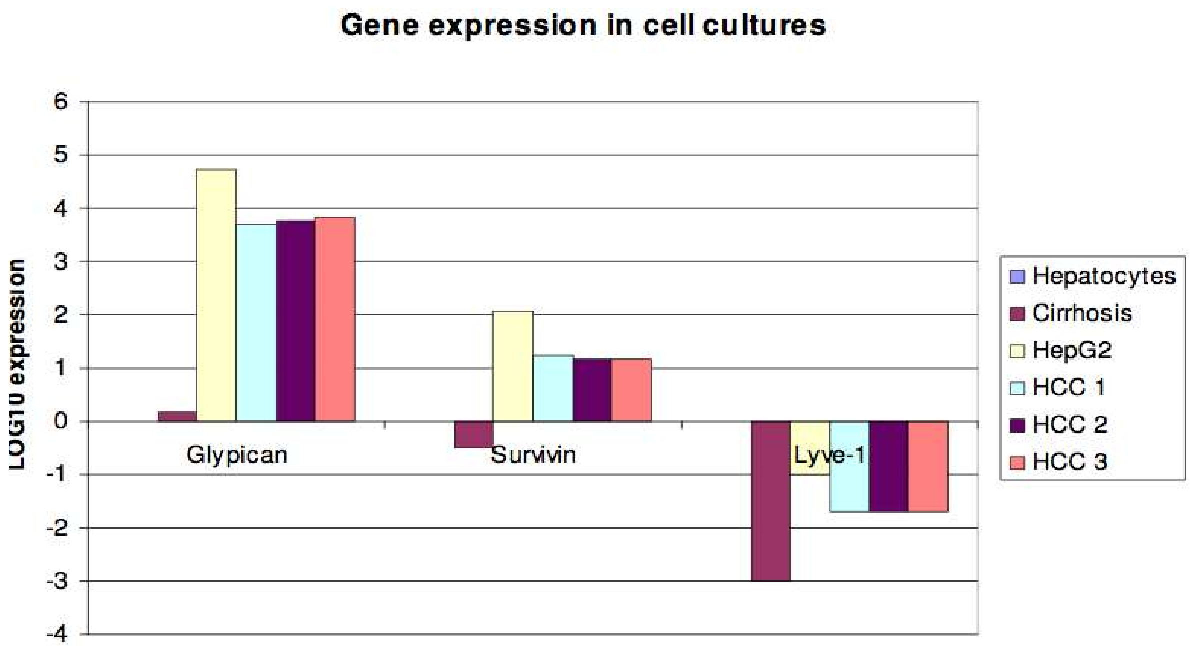
We further investigated the origin of DTC and their relationship with the bone marrow derived stem cells using gene expression profiling. Evidence from a study by Staub et al (2009) reported that the site of origin of metastatic cancers from an unknown primary can be predicted by the gene expression signatures. They showed that gene expression patterns of normal tissues harbour phenotypic information that is retained in tumours and can be sufficient to recover the type of primary tumour from expression patterns alone. This principle was applied in our study in an attempt to determine the origin of DTC.

Microarray analysis was performed to assess the expression of normal liver genes by the cells cultured from patients with HCC and compared to the HepG2 cell line, human hepatocytes (donated by the hepatocyte transplantation research group) and purified CD34^{+ve} bone marrow cells.

Quantitative RT-PCR and Microarray: Cultured cells from patients with HCC (n=3) and cirrhotic controls (n=1) were used for the experiment. Human hepatoma cell line (HepG-2), healthy human hepatocytes and purified CD34^{+ve} bone marrow cells were utilised as controls where appropriate. RT-PCR, qPCR and microarray experiments were performed according to the protocol described in section 2.9.5.

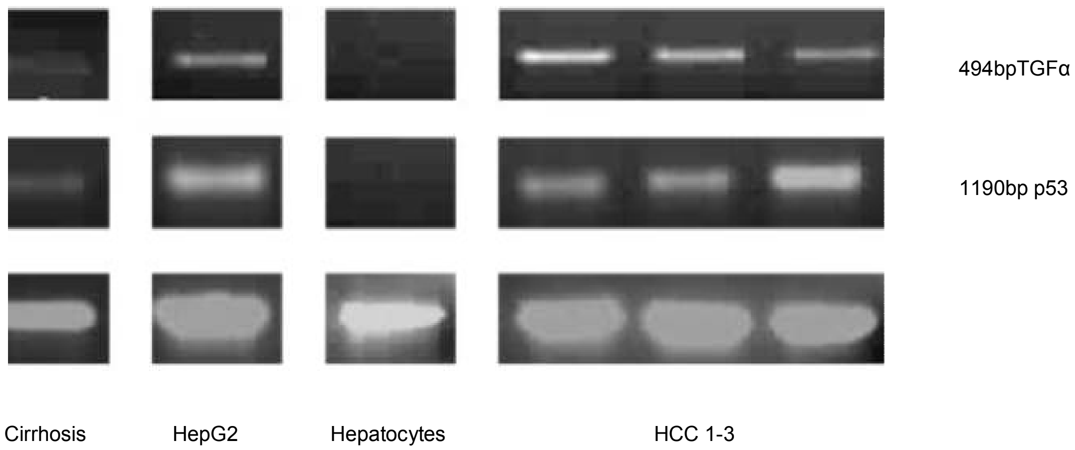
4.3 Results: qPCR comparing the expression of the markers glypican 3, survivin and LYVE1 in circulating cells from patients with HCC; HepG2 cells; primary hepatocytes and circulating cells from patients with cirrhosis but no HCC produced results in accordance with previously published findings by Llovet et al (Llovet 2006). We observed a greater than 3 logarithmic increase in the expression of glypican 3 and greater than 1 logarithmic increase in survivin expression in cells cultured from patients with HCC and in the HepG2 cell line, when compared to cells cultured from patients with cirrhosis, but not HCC and primary hepatocytes. In comparison with hepatocytes, a greater than 1 logarithmic reduction in expression of LYVE1 was observed in cells derived from patients with HCC and the HepG2 cell line, which also confirmed the findings in the published literature. The results are displayed in Figure 1.

Figure 1: Selected 3 gene expression profile in cultured cells from patients with cirrhosis and HCC (n=3) compared with controls {cells from patients with cirrhosis and no HCC (n=1); Human hepatocytes (n=1); HepG2 cell line (n=1)}



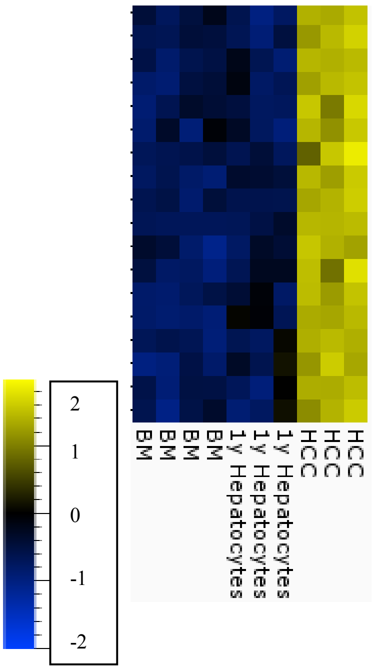
Expression of TGFα and p53 examined by RT-PCR resulted in a consistently increased expression of these markers in cells cultured from patients with HCC (n=3) or the HepG2 cell line (n=1), when compared with primary hepatocytes (n=1) and cells derived from a patient with cirrhosis, but no HCC (n=1). Ethidium bromide stained PCR after gel electrophoresis are displayed in Figure 2

Figure 2: Gel electrophoresis of PCR products p53 and TGFα expressed by cells cultured from patients with cirrhosis and HCC (n=3) compared with controls {cells from patient with cirrhosis but no HCC (n=1); human hepatocytes (n=1); HepG2 cells (n=1)}



4.3.1 Expression of cancer genes by DTC: Microarray analysis revealed an over-expression of 18 genes that are known to be associated with poor prognosis in HCC (Table 2). The results also demonstrated the over-expression of 43 genes that have been associated with poor prognosis in other solid tumours (Table 3). Of these, 23 genes have not previously been described in gene expression signatures associated with HCC and 9 are associated with adverse prognostic outcomes in HCC as well as other solid tumours. This analysis further identified increased expression of 10 genes previously described as being characteristic of HCC (Table 1). All *p*-values for differential gene expression in this analysis exceeded 1×10^{-4}. The results are presented in figures 3, 4, 5 and tables 4 and 5 respectively.

Figure 3: Microarray analysis comparing gene expression in cultured cells from patients with cirrhosis and HCC (n=3) with bone marrow cells (n=4) and primary hepatocytes (n=3) identified overexpression of 18 genes known to be associated with poor survival in HCC

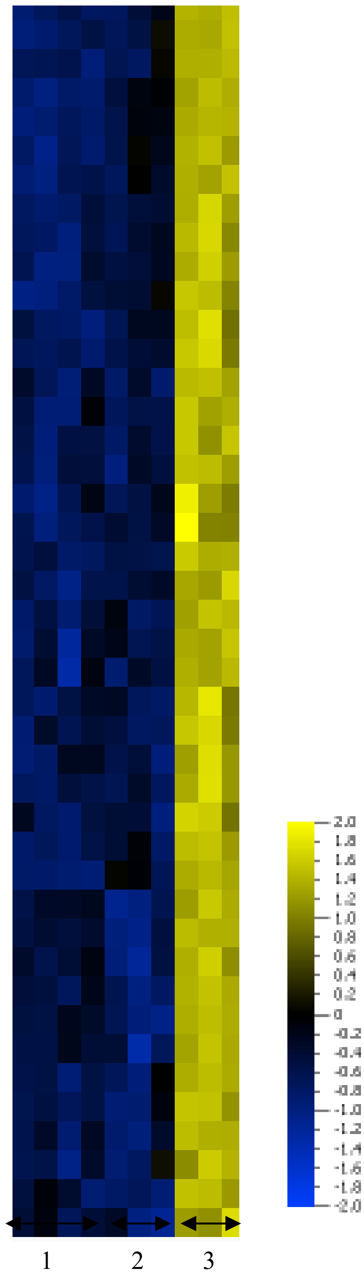


IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	2.61E-06
RAB23	RAB23, member RAS oncogene family	2.54E-06
NQO1	NAD(P)H dehydrogenase (quinone) 1	8.56E-06
ROCK2	Rho associated coiled coil containing protein kinase 2	2.66E-05
DLK1	Delta-like homolog 1	5.29E-05
ZHX2	Zinc fingers and homeoboxes 2	2.55E-04
LIN28B	Lin-28 homolog B	1.34E-04
KRT19	Keratin 19	1.99E-07
DKK1	Dickkopf homolog 1	4.00E-07
PEG10	Paternally Expressed 10	2.65E-08
CCR6	Chemokine (C-C motif) receptor 6	4.36E-05
BMP4	Bone morphogenetic protein 4	8.68E-05
ABCG2	ATP binding cassette, sub family G, member 2	1.57E-05
SPP1	Secreted phosphoprotein 1	8.02E-06
CCL20	Chemokine (C-C motif) ligand 20	2.90E-05
GSTA4	Glutathione S-transferase alpha 4	4.17E-05
ROBO1	Roundabout, axon guidance receptor, homolog 1	8.00E-05
CTGF	Connective tissue growth factor	4.29E-04

*BM- bone marrow cells; 1y hepatocytes- primary human hepatocytes; HCC- cultured cells from patients with cirrhosis and HCC

Figure 4: Microarray analysis comparing gene expression in cultured cells from patients with cirrhosis and HCC (n=3) with bone marrow cells (n=4) and primary hepatocytes (n=3) identified overexpression of 43 genes associated with poor outcome in non-HCC solid tumours (23 genes of these are not previously described in HCC)

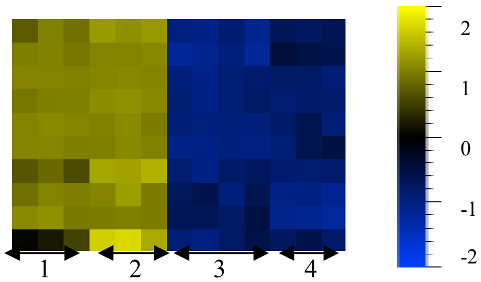
Gene symbol	Gene name	P value
S100P	S100 calcium binding protein P	2.03E-06
FBLN1	Fibulin 1	3.14E-05
CCL20	Chemokine (C-C motif) ligand 20	2.90E0-5
LAMB1	Laminin, beta 1	7.31E-07
LGR5	Leucine-rich repeat containing G protein coupled receptor 5	1.20E-06
STC2	Stanniocalcin 2	9.49E-06
MUC15	Mucin 15, cell surface associated	2.13E-05
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1	2.07E-06
ETV4	Ets variant 4	2.37E-05
KIAA1199	KIAA1199	1.98E-05
TYRO3	TYRO3 protein tyrosine kinase	3.57E-05
BMP4	Bone morphogenetic protein 4	8.68E-05
JAG1	Jagged 1	1.50E-05
EGLN3	EGL nine homolog 3	2.89E-05
ADORA2B	Adenosine A2b receptor	6.47E-05
GALNT13	GalNAc-T13	1.11E-05
PGC	Progastricsin (pepsinogen C)	2.86E-05
FDPS	Farnesyl diphosphate synthase	4.22E-04
PAGE1	P antigen family, member 1	2.42E-04
S100A1	S100 calcium binding protein A1	1.48E-07
CA9	Carbonic Anhydrase IX	1.08E-05
ROCK2	Rho associated coiled coil containing protein kinase 2	2.66E-05
MKKS	McKusick-Kaufman syndrome	1.38E-04
FAM162A	Family with sequence similarity 162, member A	4.50E-04
AHCY	Adenosylhomocysteinase	1.37E-04
DLK1	Delta-like homolog 1	5.29E-05
PTTG1IP	Pituitary tumour-transforming 1 interacting protein	1.21E-04
S100A3	S100 calcium binding protein A3	9.36E-06
CKMT1A/1B	Creatine kinase, mitochondrial 1A / 1B	1.96E-04
ABCG2	ATP binding cassette, sub family G member 2	1.57E-05
SPP1	Secreted phosphoprotein 1	8.02E-06
FAM57A	Family with sequence similarity 57, member A	4.08E-06
DSG2	Desmoglein 2	1.93E-06
ENO2	Enolase 2	3.68E-05
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3	2.61E-06
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	8.35E-07
SQLE	Squalene Epoxidase	5.53E-05
ROBO1	Roundabout axon guidance receptor, homolog 1	8.00E-05
ETV5	Ets variant 5	4.99E-05
S100A2	S100 calcium binding protein A2	3.33E-05
CTGF	Connective tissue growth factor	4.29E-04
SMYD2	SET and MYND domain containing 2	2.36E-05
RPS6KB1	Ribosomal protein S6 kinase, 70kDa, polypeptide 1	1.93E-04



*1- bone marrow cells; 2- primary human hepatocytes; 3- cultured cells from patients with cirrhosis and HCC

Figure 5: Gene expression analysis comparing cultured cells from patients with cirrhosis and HCC (n=3) with HepG2 cells (n=3), bone marrow cells (n=4) and primary hepatocytes (n=3) - The analysis identified expression of ten known diagnostic markers of HCC by the cells cultured from patients with cirrhosis and HCC.

Gene symbol	Gene name	*P* value
KRT19	Keratin 19	58.2E-10
TM4SF1	Transmembrane 4 L six, member 1	4.28E-11
AFP	Alpha-fetoprotein	6.66E-12
GPC3	Glypican 3	2.37E-11
PEG10	Paternally Expressed 10	1.23E-10
CPE	Carboxypeptidase E	8.74E-10
DKK1	Dickkopf homolog 1	5.73E-10
NEDD4L	Neural precursor cell expressed 4-like	3.72E-09
NEK3	NIMA-related kinase	3 1.67E-10
FDPS	Farnesyl diphosphate synthase	4.22E-04



*1 –cultured cells from patients with cirrhosis and HCC; 2– HepG2 cell line; 3 - bone marrow cells; 4 - primary human hepatocytes

Table 4: Genes associated with poor prognosis in solid tumours but not previously been described in HCC.

Gene symbol	Gene name
FBLN1	Fibulin 1
LAMB1	Laminin, beta 1
MUC15	Mucin 15, cell surface associated
KIAA1199	KIAA1199
TYRO3	TYRO3 protein tyrosine kinase
EGLN3	EGL nine homolog 3
GALNT13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13 (GalNAc-T13)
PAGE1	P antigen family, member 1
S100A1	S100 calcium binding protein A1
CA9	Carbonic Anhydrase IX
MKKS	McKusick-Kaufman syndrome
FAM162A	Family with sequence similarity 162, member A
AHCY	Adenosylhomocysteinase
PTTG1IP	Pituitary tumour-transforming 1 interacting protein
S100A3	S100 calcium binding protein A3
CKMT1A / CKMT1B	Creatine kinase, mitochondrial 1A / creatine kinase, mitochondrial 1B
FAM57A	Family with sequence similarity 57, member A
DSG2	Desmoglein 2
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
SQLE	Squalene Epoxidase
ETV5	Ets variant 5
S100A2	S100 calcium binding protein A2
SMYD2	SET and MYND domain containing 2

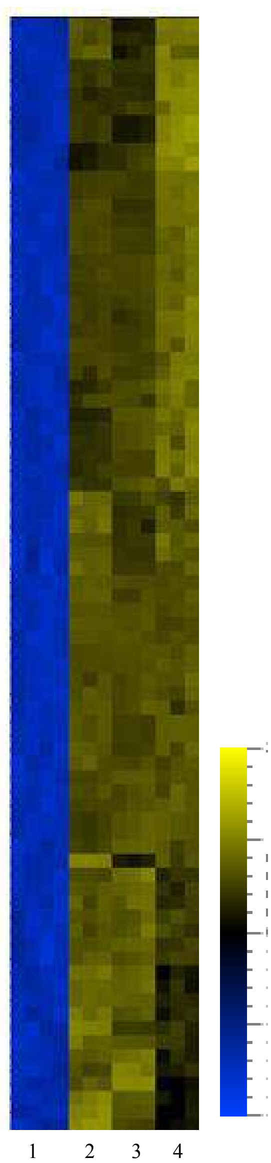
Table 5: Genes previously described in other cancers that are known markers of adverse outcomes in HCC.

Gene symbol	Gene name
S100P	S100 calcium binding protein P
LGR5	Leucine-rich repeat containing G protein coupled receptor 5
STC2	Stanniocalcin 2
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
ETV4	Ets variant 4
JAG1	Jagged 1
ADORA2B	Adenosine A2b receptor
PGC	Progastricsin (pepsinogen C)
ENO2	Enolase 2 – AKA neuron-specific enolase

4.3.2 Source of DTC in patients with HCC: Microarray analysis of high gene expression in cells cultured from patients with HCC (n=3), the HCC cell line HepG2 (n=3) and primary human hepatocytes (n=3), compared with purified CD34^{+ve} bone marrow cells from patients with no liver disease (n=4), demonstrated an expression signature characteristic of hepatocellular lineage (Figure 6). They expressed cardinal hepatocellular genes including alpha-2-macroglubulin, apolipoprotein A - C, albumin, transferrin, ceruloplasmin, fibrinogen and haptoglobin and all *p*-values for differential gene expression exceeded $1x10^{-9}$(Table 6). These results suggest that the despite the phenotypic similarities with BM stem cell, the DTC in our patient group appear to be originating from the liver tumour itself.

Figure 6: Microarray analysis of gene expression in cells cultured from patients with cirrhosis and HCC (n=3), HepG2 (n=3) and primary human hepatocytes (n=3), compared with purified CD34^{+ve} bone marrow cells (n=4), demonstrated an expression signature characteristic of hepatocellular lineage by the cells cultured from patients with cirrhosis and HCC

Gene symbol	Gene name
AZGP1	Alpha-2-glycoprotein 1, zinc-binding
F2	Coagulation factor II (thrombin)
FMO5	Flavin containing monooxygenase 5
FGB	Fibrinogen beta chain
SERPINC1	Serpin peptidase inhibitor, clade C (antithrombin), member 1
HP	Haptoglobin
C6orf145	Chromosome 6 open reading frame 145
C2 / CFB	Complement component 2 / Complement factor B
C2 / CFB	Complement component 2 / Complement factor B
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3
SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2
ORM1	Orosomucoid 1
FGG	Fibrinogen gamma chain
CP	Ceruloplasmin (ferroxidase)
FGA	Fibrinogen alpha chain
RBP4	Retinol binding protein 4, plasma
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
CFI	Complement factor I
TTR	Transthyretin
ORM1 / ORM2	Orosomucoid 1 / Orosomucoid 2
C4BPB	Complement component 4 binding protein, beta
C3	Complement component 3
CPB2	Carboxypeptidase B2 (plasma)
F5	Coagulation factor V (proaccelerin, labile factor)
APOC2	Apolipoprotein C-II
CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1
SULT2A1	Sulfotransferase family, cytosolic, 2A, DHEA-preferring, member 1
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SAA4	Serum amyloid A4, constitutive
A1CFAPOBEC1	Complementation factor
PAH	Phenylalanine hydroxylase
FGL1	Fibrinogen-like 1
ADH4	Alcohol dehydrogenase 4 (class II), pi polypeptide
MYO1B	Myosin IB
SERPIND1	Serpin peptidase inhibitor, clade D (heparin cofactor), member 1
MTTP	Microsomal triglyceride transfer protein
GPX3	Glutathione peroxidase 3 (plasma)
ANG / RNASE4	Angiogenin, ribonuclease, RNase A family, 5 / Ribonuclease, RNase A family, 4
GJB1	Gap junction protein, beta 1, 32kDa
TCEA3	Transcription elongation factor A (SII), 3
FABP1 / PRDM1	Fatty acid binding protein 1, liver / PR domain containing 10
ADH6	Alcohol dehydrogenase 6 (class V)
TF	Transferrin
SERPINA7	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7
APOA2	Apolipoprotein A-II



APOH	Apolipoprotein H (beta-2-glycoprotein I)
ALB	Albumin
AMBP	Alpha-1-microglobulin/bikunin precursor
EPB41L5	Erythrocyte membrane protein band 4.1 like 5
SLC47A1	Solute carrier family 47, member 1
APOA1	Apolipoprotein A-I
UGT2B7UDP	Glucuronosyltransferase 2 family, polypeptide B7
PVRL3	Poliovirus receptor-related 3
APOB	Apolipoprotein B (including Ag(x) antigen)
A2M	Alpha-2-macroglobulin
FAM114A1	Family with sequence similarity 114, member A1
HGD	Homogentisate 1,2-dioxygenase (homogentisate oxidase)
IGF2 / INS-IGF2	Insulin-like growth factor 2 (somatomedin A) / INS-IGF2 readthrough transcript
KIF21A	Kinesin family member 21A
FN1	Fibronectin 1
PARD3	Par-3 partitioning defective 3 homolog
MLLT4	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog); translocated to, 4
TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)
ENPP1	Ectonucleotide pyrophosphatase / phosphodiesterase 1
AHSG	Alpha-2-HS-glycoprotein
DSP	Desmoplakin
SEP10	Septin 10
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1
SDC2	Syndecan 2
EMP2	Epithelial membrane protein 2
SH3D19	SH3 domain containing 19
PTPRK	Protein tyrosine phosphatase, receptor type, K
PPIC	Peptidylprolyl isomerase C (cyclophilin C)
SCML1	Sex comb on midleg-like 1
PECR	Peroxisomal trans-2-enoyl-CoA reductase
RNF128	Ring finger protein 128
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein

*1-bone marrow cells; 2- cells cultured from patients with cirrhosis and HCC; 3- human hepatoma cells line (HepG2); 4- primary human hepatocytes.

Table 5 : List of genes and *P*-values from a microarray analysis comparing gene expression in bone marrow (n=4) with circulating HCC cells (n=3); HepG2 cells (n=3) and primary hepatocytes (n=3).

Gene symbol	**Gene name**	***P*-Value**
AZGP1	Alpha-2-glycoprotein 1, Zinc-binding	9.32E-12
F2	Coagulation factor II (thrombin)	3.23E-12
FMO5	Flavin containing monooxygenase 5	3.34E-09
FGB	Fibrinogen beta chain	7.19E-14
SERPINC1	Serpin peptidase inhibitor, clade C (antithrombin), member 1	4.45E-11
HP	Haptoglobin	4.34E-12
C6orf145	Chromosome 6 open reading frame 145	5.22E-10
C2 / CFB	Complement component 2 / Complement factor B	9.32E-10
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	7.34E-12
SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2	1.81E-09
ORM1	Orosomucoid 1	6.70E-12
FGG	Fibrinogen gamma chain	2.92E-13
CP	Ceruloplasmin (ferroxidase)	1.03E-13
FGA	Fibrinogen alpha chain	1.30E-12
RBP4	Retinol binding protein 4, plasma	7.76E-11
PPARGC1A	Peroxisome proliferator-activated receptor gamma, co activator 1 alpha	8.88E-12
CFI	Complement factor I	6.45E-13
TTR	Transthyretin	5.83E-11
ORM1/ ORM2	Orosomucoid 1 / Orosomucoid 2	4.27E-11
C4BPB	Complement component 4 binding protein, beta	5.62E-10
C3	Complement component 3	6.96E-12
CPB2	Carboxypeptidase B2 (plasma)	1.07E-10
F5	Coagulation factor V (proaccelerin, labile factor)	1.49E-09
APOC2	Apolipoprotein C-II	5.71E-10

CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1	7.16E-10
SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	1.88E-10
SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1.80E-10
SAA4	Serum amyloid A4, constitutive	1.56E-10
A1CF	APOBEC1 complementation factor	2.95E-10
PAH	Phenylalanine hydroxylase	4.31E-09
FGL1	Fibrinogen-like 1	2.24E-11
ADH4	Alcohol dehydrogenase 4 (class II), pi polypeptide	2.34E-10
MYO1B	Myosin IB	7.15E-10
SERPIND1	Serpin peptidase inhibitor, clade D (heparin cofactor), member 1	4.46E-10
MTTP	Microsomal triglyceride transfer protein	1.13E-09
GPX3	Glutathione peroxidase 3 (plasma)	4.24E-11
ANG/ RNASE4	Angiogenin, ribonuclease, RNase A family, 5 / Ribonuclease, RNase A family, 4	3.16E-10
GJB1	Gap junction protein, beta 1, 32kDa	3.40E-09
TCEA3	Transcription elongation factor A (SII), 3	3.71E-09
FABP1/ PRDM10	Fatty acid binding protein 1, liver / PR domain containing 10	1.75E-13
ADH6	Alcohol dehydrogenase 6 (class V)	2.26E-11
TF	Transferrin	1.04E-11
SERPINA7	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	4.93E-12
APOA2	Apolipoprotein A-II	2.43E-10
APOH	Apolipoprotein H (beta-2-glycoprotein I)	1.60E-12
ALB	Albumin	4.28E-13
AMBP	Alpha-1-microglobulin / bikunin precursor	6.22E-10
EPB41L5	Erythrocyte membrane protein band 4.1 like 5	9.23E-10
SLC47A1	Solute carrier family 47, member 1	1.98E-09
APOA1	Apolipoprotein A-I	1.45E-14
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	9.19E-10

PVRL3	Poliovirus receptor-related 3	9.81E-10
APOB	Apolipoprotein B (including Ag(x) antigen)	5.32E-13
A2M	Alpha-2-macroglobulin	1.78E-10
FAM114A1	Family with sequence similarity 114, member A1	1.14E-10
HGD	Homogentisate 1,2-dioxygenase (homogentisate oxidase)	4.05E-12
IGF2/INS-IGF2	Insulin-like growth factor 2 (somatomedin A) / INS-IGF2 readthrough transcript	1.66E-10
KIF21A	Kinesin family member 21A	3.75E-10
FN1	Fibronectin 1	7.18E-11
PARD3	Par-3 partitioning defective 3 homolog	3.60E-09
MLLT4	Myeloid/lymphoid or mixed-lineage leukaemia; translocated to, 4	2.65E-09
TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)	3.05E-09
ENPP1	Ectonucleotide pyrophosphatase / phosphodiesterase 1	3.60E-09
AHSG	Alpha-2-HS-glycoprotein	1.59E-09
DSP	Desmoplakin	2.42E-10
SEP-10	Septin 10	9.51E-10
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	6.51E-14
SDC2	Syndecan 2	3.49E-09
EMP2	Epithelial membrane protein 2	7.73E-10
SH3D19	SH3 domain containing 19	2.55E-09
PTPRK	Protein tyrosine phosphatase, receptor type, K	1.01E-10
PPIC	Peptidylprolyl isomerase C (cyclophilin C)	3.51E-09
SCML1	Sex comb on midleg-like 1 (Drosophila)	3.57E-10
PECR	Peroxisomal trans-2-enoyl-CoA reductase	2.03E-10
RNF128	Ring finger protein 128	8.69E-10
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.05E-09

4.4 Discussion: Disseminated tumour cells have been identified in the blood and bone marrow of patients with breast, colorectal, oesophageal and prostate cancers. Their presence has been associated with tumour recurrence as well as poor long term survival. Several studies have suggested that DTC can be used as a surrogate marker of aggressive tumour biology, however, the true malignant nature of these cells has not been demonstrated. The isolation of DTC in peripheral blood samples of breast cancer patients 22 years after curative mastectomy highlights the need to study their biology and understand the molecular pathways associated DTC regulation.

Understanding of the role of DTC in HCC is limited and their role in the development of tumour metastases is not clear. In the previous chapter we developed a protocol and successfully cultured DTC from patients with cirrhosis and HCC. The experiment also isolated similar cells from patients with liver cirrhosis and no HCC. Immunocytochemistry analysis showed that cells from both groups expressed morphological and functional markers similar to hepatocytes. This led to the work in this chapter using molecular assays to identify potential differences between the cultured cells from patients with cirrhosis and HCC and those with cirrhosis but no HCC.

Accumulation of mutations and altered expression of various cell cycle regulatory genes result in carcinogenesis. A variety of tumour suppressor genes (P53, RB and IGF2R) and oncogenes (c-myc, CCND1, CTNNB1, and c-Met) along with activation of the matrix metalloproteinase (MMP) family, angiopoietin, and vascular endothelial growth factor (VEGF) and inactivation of E-cadherin have been demonstrated to play pivotal roles in the development and progression of HCC. A comprehensive expression analysis of this complex process using microarray technology has great potential for the discovery of new genes involved in carcinogenesis, cDNA microarray analysis for HCC was first reported by Lau et al (2000). Since this publication numerous microarray studies have been carried out in an effort to elucidate molecular mechanisms of hepatocarcinogenesis, metastasis and/or prognosis, however, reliable clinical markers for HCC have yet to be defined.

Studies that focussed on identifying diagnostic biomarkers for HCC have produced varying results. Chuma et al(2003) suggested heat shock protein 70 (HSP70), Capurro and Nakatsura et al (2003) supported glypican-3 (GPC3) and telomerase reverse transcriptase (TERT) while Miura and Smith et al (2003) concluded serine/threonine kinase (STK6), and phospholipase A2 (PLAG12B) were reliable markers of hepatocarcinogenesis. A molecular index using a 13-gene set (including TERT, TOP2A, and PDGFRA) (Paradis 2003) followed by a more recent microarray generated a signature of 120 genes which has been used to discriminate dysplastic nodules from HCC in hepatitis B patients with chronic liver disease (Nam 2005). Unfortunately none of these studies have produced markers that are sufficiently acceptable for a molecular diagnosis of HCC (Bruix 2005).

Llovet et al (2006) in their study assessed the transcriptional profiles of 55 candidate genes in dysplastic nodules and HCC and identified a 3 gene signature (GPC3, LYVE1 and Survivin) that has a 94% accuracy in the diagnosis of HCC. The results from our qPCR experiment using this 3-gene signature and the RT-PCR using TGFα and p53 demonstrated that only cultured cells from patients with cirrhosis and HCC have malignant characteristics comparable with human hepatoma cell line HepG2. The cultured cells from patients with HCC were further analysed for the expression of neoplastic genes using microarray assays. Small number of cells from patients with cirrhosis but no HCC limited their inclusion in the analysis and use as controls.

Weinberg et al proposed six characteristics of cancer that enable tumour growth and metastasis, that include: 1) sustaining proliferative signalling 2) evading growth supressors 3) activating invasion and metastasis 4) enabling replicative immortality 5) inducing angiogenesis and 6) resisting cell death. More than 300 studies have been published identifying dysregulated genes in HCC. The studies targeted one or more sub-groups of the carcinogenesis pathway described by Weinberg et al (2011). Although these studies identified a wide spectrum of adverse genes the results were, however, inconsistent. For the purpose of our study we have highlighted those genes with the best supporting evidence that have a role in the development and progression of HCC.

Proliferation cluster genes are required for cell cycle progression. Studies investigating this group of genes in HCC identified that their enhanced expression is associated with aggressive tumours (vascular invasion) and poor prognosis (Chen 2002, Thorgeirsson 2004, Segal 2004, Yu 2005). The common genes highlighted in these studies include CCNA2, CCNB2, CDC2, CKS2, E2F3, FOXMI, IGF2BP3, PCNA, PLK1, TOP2A.

The second group of genes explored were the hepatic progenitor specific and EpCAM-positive gene signatures. Recent studies suggest that EpCAM positive cells are tumour initiating or cancer stem cells. Yamashida et al showed that EpCAM-positive cells isolated from human HCCs self-renewed, differentiated *in vitro* and formed large tumours in NOD/SCID mice (Yamashida 2009). The EpCAM positive gene signature is comprised of 70 genes and exhibits increased expression of marker genes characterizing hepatic progenitors. These include cytokeratin 19 (CK19), C-kit and Wnt signalling-induced genes DKK1 and BAMBI. The common genes identified in the published literature are; CK19, CK8, CK18, EpCAM, CD133, AFP, MYC, DLK1, DKK1. (Lee 2006, Yamashita 2008). EpCAM expression is linked to poor prognosis in HCC. A recent review of gene signatures in HCC by Andrisani et al (2011) concluded that the proliferation cluster and hepatic cancer stem cell gene signatures are the only groups that have potential prognostic value in the clinical management of HCC. The results of our analysis demonstrated over expression of such genes in cells cultured from patients with HCC. They also over expressed other genes involved in HCC progression and metastasis described in the literature. We also noted that our *in vitro* cultured cells from patients with HCC demonstrated abnormal expression of 43 genes that correlate with poor outcome in non-HCC solid tumours, and of note 23 of these genes that have not been described previously in HCC.

Molecular analysis was performed to further characterise cells cultured from patients with HCC and those with cirrhosis, but without HCC. The results demonstrated that although the cells cultured from these two groups share some morphological and functional similarities they are inherently different. The 3 gene signature showed that only cells disseminated into the peripheral blood of patients with HCC expressed markers of HCC. These cells may represent primary tumour cells and therefore could be used to understand tumour biology and also develop new treatment strategies. Unfortunately due to poor *in vitro* expansion of cultured cells from patients with cirrhosis but without HCC, these cells could not be included in the microarray analysis.

The results from microarray analysis not only supported the neoplastic nature of DTC, but also provided further biological information about these cells. Sustaining proliferative signalling is the most important trait of cancer cells and this process is mediated by growth factors that regulate the cell cycle. Cell migration and invasion of tumour cells is further facilitated by a group of genes which are collectively known as EPCAM genes. The DTC in our experiment expressed both potentially important hallmarks of cancer and genes indicating biological aggressiveness. Several cancer pathways including the Wnt cell cycle pathway and genes involved in the epigenetic regulation with frequent genetic alterations have emerged as oncogenic drivers in HCC. Further understanding of the specific pathways associated with DTC will help understand the pathogenesis of HCC recurrence and may pave the way for the development of targeted treatments.

4.4.1 Origin of DTC: There is conflicting evidence regarding the role of bone marrow derived stem cells in liver regeneration and hepatocarcinogenesis. The DTC in our experiment expressed markers similar to BM stem cells suggesting the possibility that these represented circulating BM derived stem cells. To investigate this we utilised the principle described by Staub et al. They demonstrated that tumours retain a significant part of their normal phenotype that is characteristic of their tissue origin and microarray-based tissue-type classifiers trained solely on normal tissues can predict their origin. The microarray analysis of cells cultured from HCC demonstrated the expression of genes that are characteristic of hepatocellular lineage, thereby excluding the presence of BM derived stem cells.

4.5 Conclusion: From the results of this chapter we conclude that the cells cultured from patients with cirrhosis and HCC express a malignant phenotype. The circulating cells are hepatocellular in origin and may represent an aggressive HCC.

The aim of next chapter is to develop a suitable animal model to study the *in vivo* behaviour of DTC.

CHAPTER 5: ANIMAL MODEL TO STUDY THE *IN VIVO* BEHAVIOUR OF CELLS CULTURED FROM PATIENTS WITH HCC

5.1 Introduction: DTC have been detected in blood and bone marrow samples of patients with HCC using immunocytochemistry and molecular assays. The presence of DTC has been associated with tumour recurrence and poor prognosis (Kamiyama 2006, Marubashi 2007). However, variations in methodology and lack of knowledge about the true functional and biological behaviour of micrometastatic cells have prevented the transfer of these investigations into clinical practice. Studies focusing on the pattern of tumour recurrence in patients with detectable DTC have produced variable results and some including our previous study questioned the malignant potential of DTC (Sutcliffe 2005, Pantel 2009). Our previous *in vitro* experiment demonstrated that DTC can be isolated from a small sub-group of patients with HCC and that these cells express a malignant phenotype. Although these results generated some useful information about the nature of DTC, translating these findings into clinical practice demands a suitable small animal model to study their *in vivo* biological behaviour. To date the *in vivo* malignant potential of the DTC in HCC has not been effectively demonstrated. The aim of this chapter was to develop an effective animal model and to study the *in vivo* behaviour of the cultured cells from both patients with and without HCC.

5.2 Materials and methods:

Development of a mouse model: Successful transplantation of human hepatocytes into an animal model requires it not to reject the graft and to provide a "supportive niche" that promotes expansion and engraftment of the transplanted cells (Meuleman 2005, Weber 2009). Recipient mice that have an inborn (genetic) or acquired (drug induced) immune deficiency have been extensively used as hosts to study the *in vivo* behaviour of hepatocytes as well as hepatoma cell lines.

For the purpose of our study the severe combined immunodeficiency (SCID) mouse was selected as our animal model. SCID is due to a rare recessive mutation on Chromosome 16 that results in failure of the development of cellular and humoral immune systems. SCID mice, therefore, have both T and B lymphocyte deficiency making them excellent recipients for cell transplantation. The SCID mouse model has been useful to study the potential use of hepatocyte cell transplantation as alternative to liver transplantation (Fisher 2006). Despite early success, improving and stabilizing engraftment and repopulation of transplanted human hepatocytes remains a significant obstacle. The success of hepatocyte repopulation in the host tissue is strongly dependent on the volume of cells used and the mode of infusion.

The liver is an optimal site for transplantation of hepatocytes due to the presence of a physiological matrix that aids repopulation. The potential drawback with this mode of infusion is the difficulty in localizing the transplanted cells. Studies have shown that in the rodent hepatic remodeling takes place in 3-7 days making the engrafted cells histologically indistinguishable from the host cells (Allen, 2001). Extra hepatic sites such renal capsular space, peritoneal cavity, spleen and foot pad have been used as sites of infusion. Although they cannot offer a hepatic micro-environment, their use has the potential advantage of better localization of transplanted cells on a non-hepatic background.

Studies with human hepatoma cell lines have used large number of cells (ranging from 1-5 million) in both hepatic and extra-hepatic locations (Scatton 2006, 2007, Mischek 2009). The minimum cell number for the initiation of neoplastic transformation in the host organs has not been defined. The median cell mass obtained in a selected group of patients with cirrhosis and

HCC and cirrhosis, but without HCC from our previous experiment was 2x 10^4 cells (Range - 2-6 x10^4 cells).

To study the *in vivo* behaviour of cells when introduced in small number and to localize them in the host tissue accurately, we initially used human hepatoma cell line (HepG2). To facilitate localization, HepG2 cells were injected into the renal sub-capsule of SCID mice.

5.2.1 Section 1: Development of a transplant model:

Six week old male SCID mice were purchased from Charles River UK and handled in accordance with Home Office UK guidelines. The experiment was performed using the relevant protocols described in chapter 2. Briefly, frozen human hepatoma cells were thawed and cultured overnight in T75 flasks. Cells were detached, washed and aliquots of 10^4,10^5&10^6 Hep G2 cells were prepared for injection. Nine mice were anaesthetized and the described cell volumes were injected into the renal sub-capsular space of the left kidney.

The mice from each group were sacrificed at 2, 4 and 6 weeks and the kidney specimens were collected for analysis. 10µm cryosections were prepared and the slides were analysed using H&E staining and immunohistochemistry. Cam 5.2 (CK8 and 18), MHC class-1 and Ki67 antibodies were used to localize the transplanted cells, initial test with these antibodies confirmed no cross-reactivity with mouse tissue. The results are shown in Figures 1, 2, 3 and 4.

Laser capture microdissection was attempted in selected samples. To facilitate the use of laser capture microdissection, cryosections were mounted on a special membrane slides and air dried overnight. The dissected tissue sections are shown in Figure 5.

Results:

Figure 1a – H&E section (10 X magnification) – Injected HepG2 cells localised in the kidney sub-capsular space

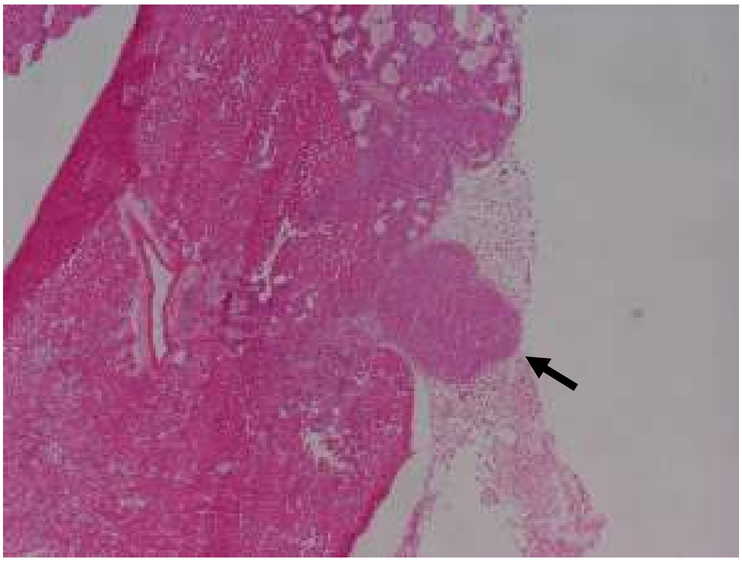


Figure 1b -H&E section (50X magnification) - Injected HepG2 cells in the kidney sub-capsular space

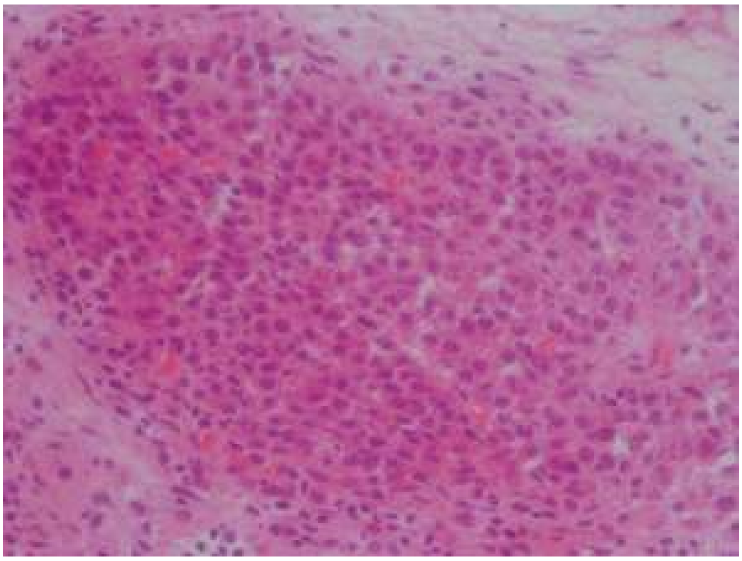


Figure 2a- Injected HepG2 cells localised using immunofluorescence staining with antibody against cytokeratin 8 and 18 (Cam 5.2) - The HepG2 cells stained positive (thick arrow) on a background negative staining of mouse kidney tissue (thin arrow).

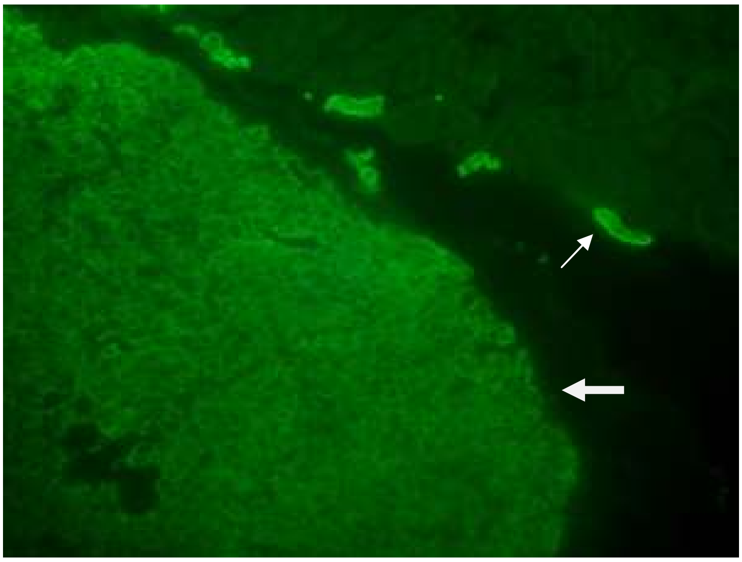


Figure 2b- Positive control –Human liver stained positive with Cam5.2.

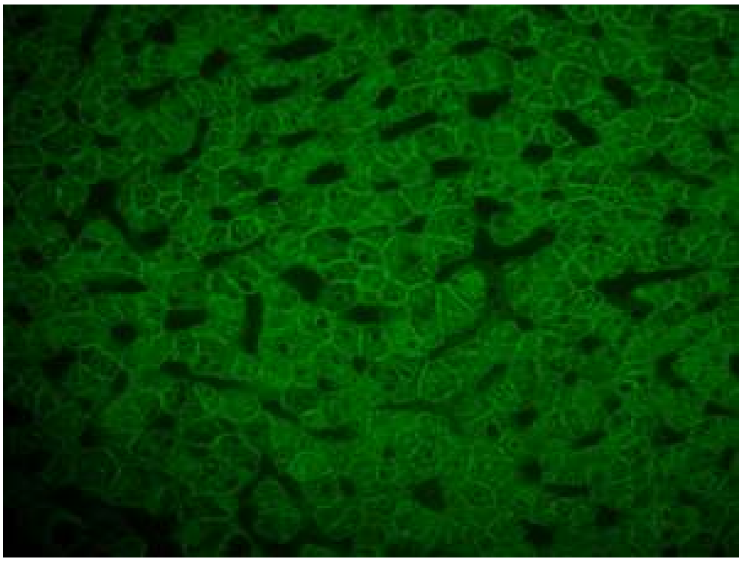


Figure 3a- Injected HepG2 cells localised using antibody against human MHC class-1 - The HepG2 cells stained positive (thick arrow) on a background negative staining of mouse kidney tissue (thin arrow).

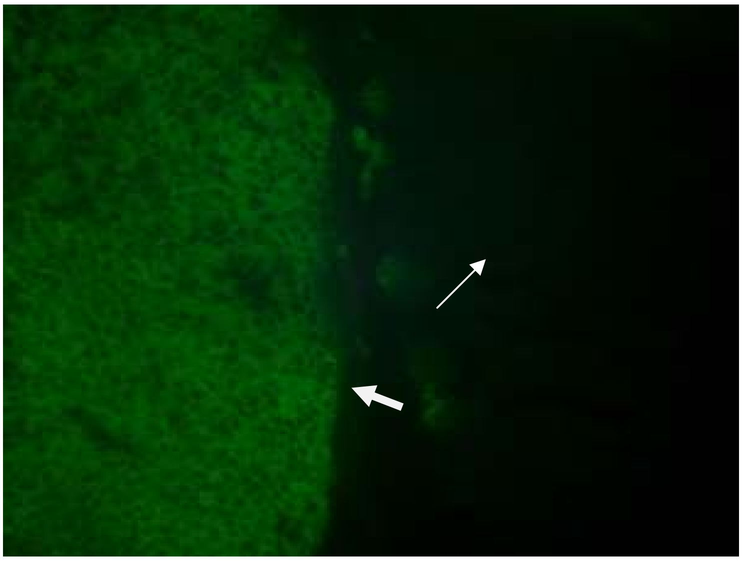


Figure 3b- Positive control- MHC class-1staining of human liver tissue

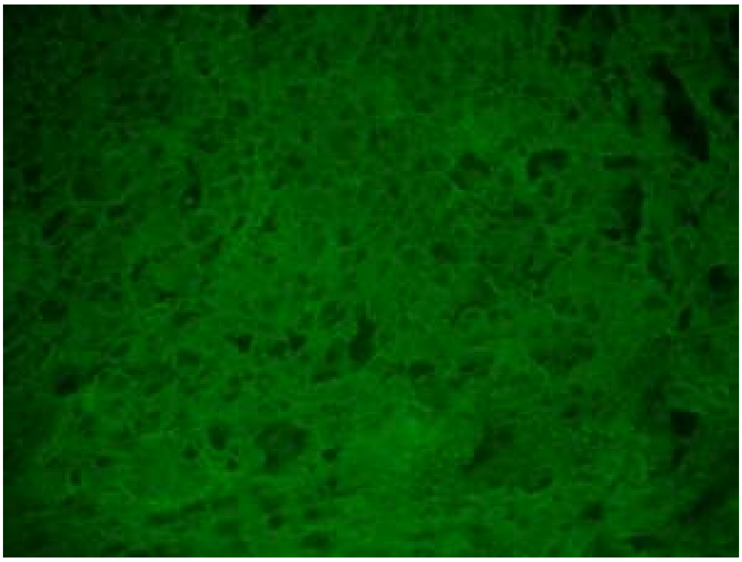


Figure 4- Cryosections stained with cell proliferative marker Ki 67- A positive staining indicating that the injected hepatoma cells can proliferate in the host tissue leading to tumour formation.

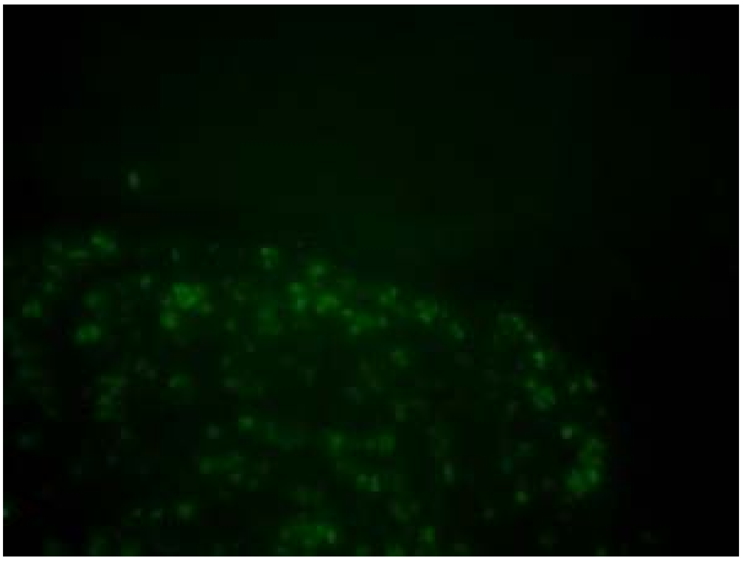


Figure 5a: Use of laser microdissection- The tissue sections were mounted on a special membrane slide and the desired cell group was dissected using a laser beam.

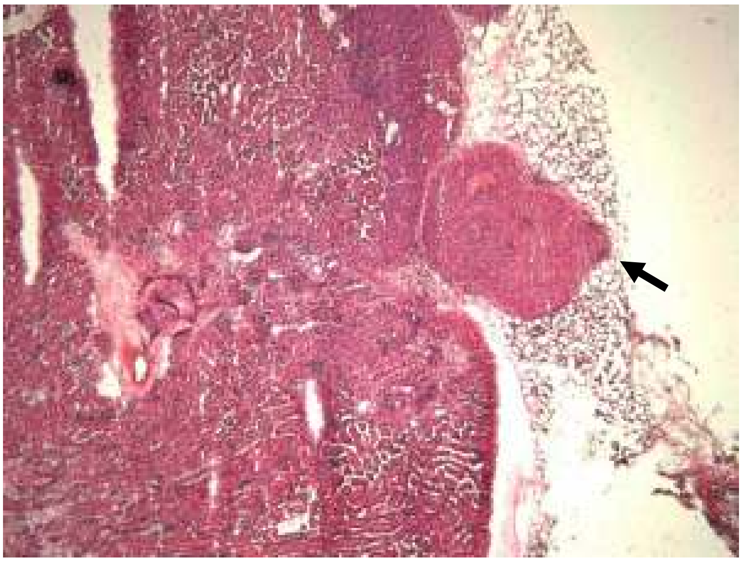


Figure 5b: Dissected tissue section

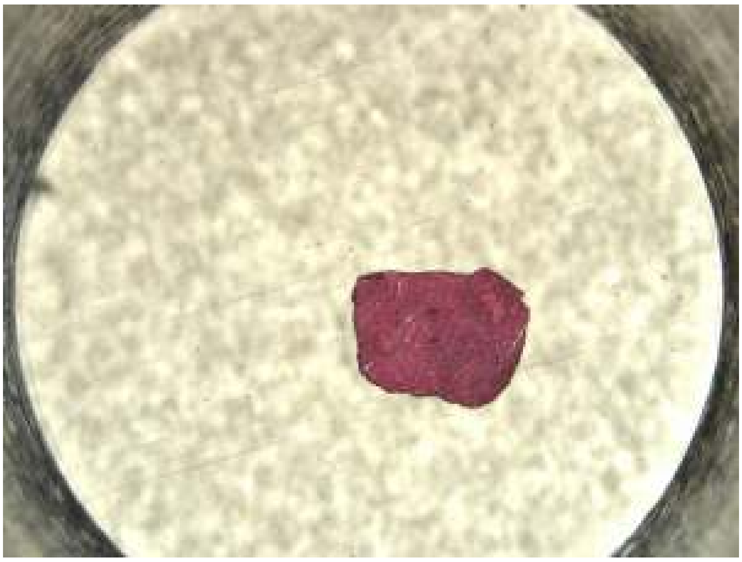
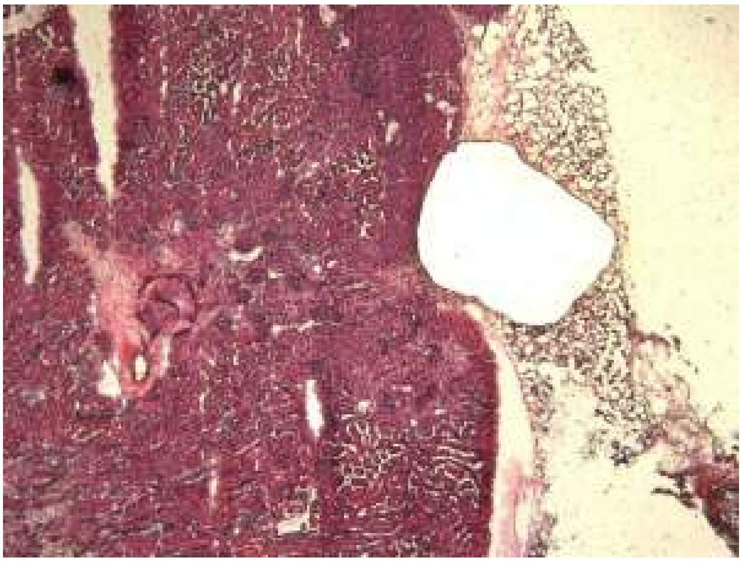


Figure 5c: Tissue section following removal of the desired cell cluster using laser microdissection



The results of the tissue analysis according to the cell volume and period of incubation are presented in a tabular format.

Table 1) Mice sacrificed following 2 week incubation (n=3)

HepG2 cell volume	Macroscopic appearance of the kidney	Microscopy (H&E and Immunohistochemistry)			
		H&E	Cam 5.2	MHC class-1	Ki 67
10^4 cells	Normal	Equivocal	Positive	Positive	Negative
10^5 cells	Normal	Detectable HepG2 cells	Positive	Positive	Equivocal
10^6 cells	Normal	Detectable HepG2 cells	Positive	Positive	Equivocal

Table 2) Mice sacrificed following 4 week incubation (n=3)

HepG2 cell volume	Macroscopic appearance of the kidney	Microscopy (H&E and Immunohistochemistry)			
		H&E	Cam 5.2	MHC class-1	Ki 67
10^4 cells	Normal	Equivocal	Positive	Positive	Negative
10^5 cells	Normal	Detectable HepG2 cells	Positive	Positive	Positive
10^6 cells	Normal	Detectable HepG2 cells	Positive	Positive	Positive

Table 3) Mice sacrificed following 6 week incubation (n=3)

HepG2 cell volume	Macroscopic appearance of the kidney	Microscopy (H&E and Immunohistochemistry)			
		H&E	Cam 5.2	MHC class-1	Ki 67
10^4 cells	Normal	Equivocal	Positive	Positive	Negative
10^5 cells	Normal	Detectable HepG2 cells	Positive	Positive	Positive
10^6 cells	Visible tumour	Detectable HepG2 cells	Positive	Positive	Positive

Discussion: Several studies were undertaken to identify an ideal mouse organ for hepatocyte transplantation. As the liver represents an ideal "home" for transplanted hepatocytes, the majority of the reported studies used either direct hepatic injection or splenic and portal venous routes for cell transplantation. The limitation with this mode of transplantation was the difficulty in localising the injected hepatocytes in the host liver background. This consequently led to exploration of ectopic sites such as kidney capsule, subcutaneous space and interscapular fat pads for hepatocyte transplantation. The results demonstrated that these sites can support short-term hepatocyte survival despite the apparent lack of hepatotrophic factors.

The second issue with cell transplantation was poor engraftment, the maximum percentage of engraftment that can be achieved is of the order of 2-5% even when large cell numbers are injected (>10^6 cells). The results from studies using the liver for cell transplantation showed that more than 90% of transplanted hepatocytes are not detectable in the host liver following infusion (Allen 2001). There is also evidence to suggest that natural killer cell mediated lysis plays an important role in eliminating allogenic cells in SCID mice even in the absence of T and B lymphocytes (Olszewski 1999, Kawahara 2010).

The results from our experiment show that although micro and macroscopic neoplastic changes were detectable only from injected cell numbers $>10^5$, HepG2 cells from smaller cell number injections (10^4 cells) persisted in the host tissue after 2 and 4 weeks. The persistence of HepG2 cells even from very small cell mass injections suggest that malignant hepatic cells have mechanisms to survive in ectopic tissue and to escape cell lysis by the host natural killer cells. The latter is an important property for tumour propagation. The successful localisation of small cell number injections led to the final experiment of injecting cultured cells into an animal model to study their *in vivo* behaviour.

5.2.2 Section 2: SCID mouse model to study the *In vivo* properties of cultured cells.

Materials and methods:

In vitro cultures from patients with cirrhosis and HCC (n=6)

In vitro cultures from patients with cirrhosis and without HCC (n=3)

Primary healthy human hepatocytes (n=6)

Cell sample preparation: The experimental cells were thawed, purified and prepared for transplantation following the protocol described in Chapter 2.

Fifteen mice were randomly allocated into cell transplant experimental and control groups. *In vitro* cultures from patients with HCC (n=6), cirrhosis, but no HCC (n=3) and normal human hepatocytes were used with the latter two as controls. The cultured cells from patients with cirrhosis and HCC were injected into the renal sub-capsule (n=3) and directly into the left lobe of the liver (n=3). $2x10^4$ cells from each group were injected into the liver or kidney of SCID mice using the protocols described in Chapter 2. Cells cultured from patients with cirrhosis, but no HCC were limited in number and therefore were used only in the renal sub-capsular space.

Briefly, under anaesthesia, the abdominal cavity was accessed by a laparotomy and the organ of choice (liver or kidney) was exposed. The prepared cell number was injected directly into the left lobe of the liver or lower pole of the left kidney using a Hamilton syringe. Three aliquots of cultured cells from patients with cirrhosis and HCC were injected into the mouse liver using human primary hepatocytes as controls (n=4). For the kidney injections a total of eight SCID mice were used as follows; 3 with cells cultured from patients with cirrhosis and HCC, 3 with cells cultured from patients with liver cirrhosis and without HCC and 2 with normal primary human hepatocytes.

Retrieving transplanted organs: The mice were sacrificed by dislocating the cervical vertebrae. SCID mice in which the liver was used as the site of injection were sacrificed after 4 weeks. The liver was retrieved and the cell transplant lobe was excised from the whole organ. The mice with renal sub-capsular injections were sacrificed after 2 weeks, both kidneys were retrieved in total but the left kidney was then divided transversely to obtain separate upper and lower poles. The individual portions of tissue were transported to the laboratory in ice cold UW solution and stored in labelled cryo-vials at -80°C following snap freezing.

The samples were either processed and embedded in paraffin wax or frozen tissue was directly sliced into 10 µm cryosections for further analysis. The host tissue sections were analysed for the presence of any neoplastic changes as well as persistence of injected cultured cells. Immunohistochemistry, H&E and genomic human DNA analysis were used as tools for the analysis. Antibodies specific to human cytokeratin (CK8&18 pre-diluted by the manufacturer), MHC class-1(1:250 dilution) and CD34 (1:250 dilution) were selected for immunohistochemistry. The primary antibodies were diluted in 1% PBS and the optimal concentration was determined by serial dilution.

The paraffin or frozen tissue samples were processed using the protocols described in Chapter 2 and the results were reviewed by 3 'blinded' liver histopathologists.

Results:

Macroscopic appearance:

There were no noticeable gross macroscopic changes in the transplanted kidney or liver specimens following retrieval.

Microscopic appearance:

Analysis of liver sections: The H&E staining of liver sections obtained from mice that were injected with cells from patients with cirrhosis and HCC showed focal dysplastic changes and/or features of neoplasia with anisocytosis (cells of unequal size), poikilocytosis (abnormally shaped cells), hyperchromatism and lobular disarray The liver sections from relevant controls (primary human hepatocytes) demonstrated a normal liver architecture on H&E staining.

Figure 6a- H&E staining of SCID mouse liver injected with cells cultured from patients with cirrhosis and HCC – Abnormal cells with anisocytosis, poikilocytosis, hyperchromatism and lobular disarray.

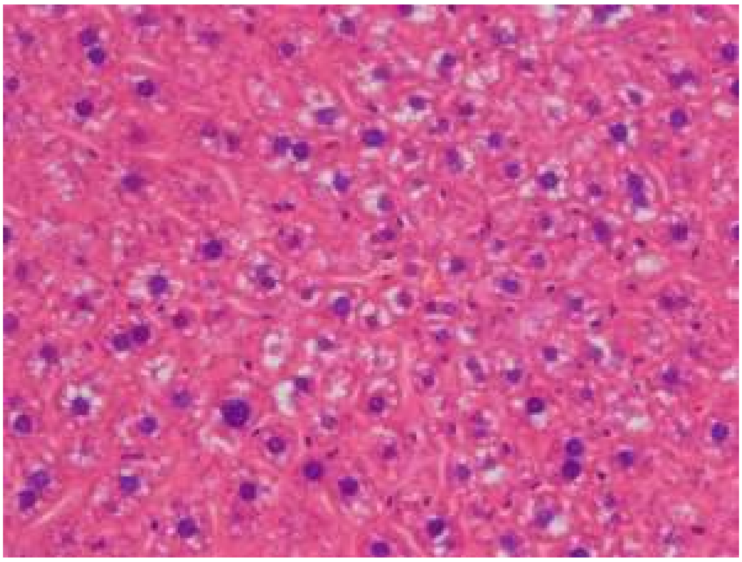
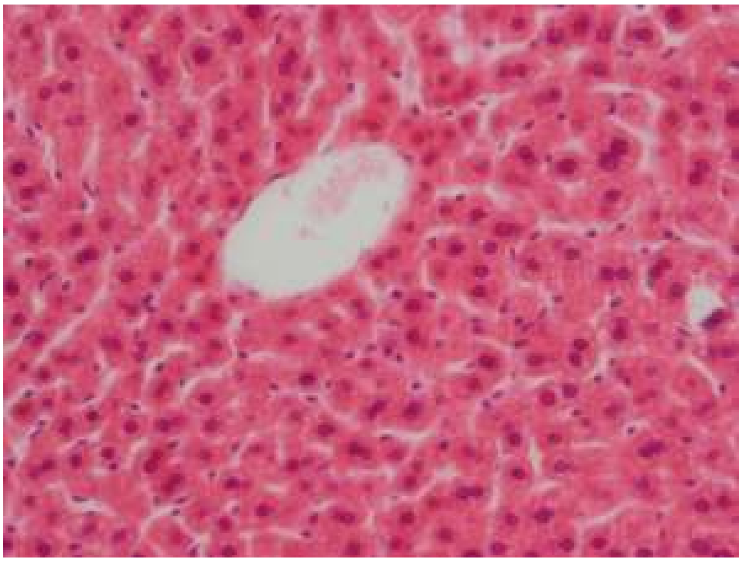


Figure 6b: H&E staining of SCID mouse liver injected with primary human hepatocytes- Normal architecture with none of the above mentioned changes



The immunohistochemistry analysis was limited only to CD34 antibody due to cross reaction between the mouse background liver and human specific antibodies (MHC class-1 and CK8&18). The CD34 antibody analysis confirmed that the neoplastic cells that were evident on the H&E sections were of human origin.

Figure 7a- Immunohistochemistry using antibody against CD34- Positive staining of the endothelial cells in a section of human liver (arrow).

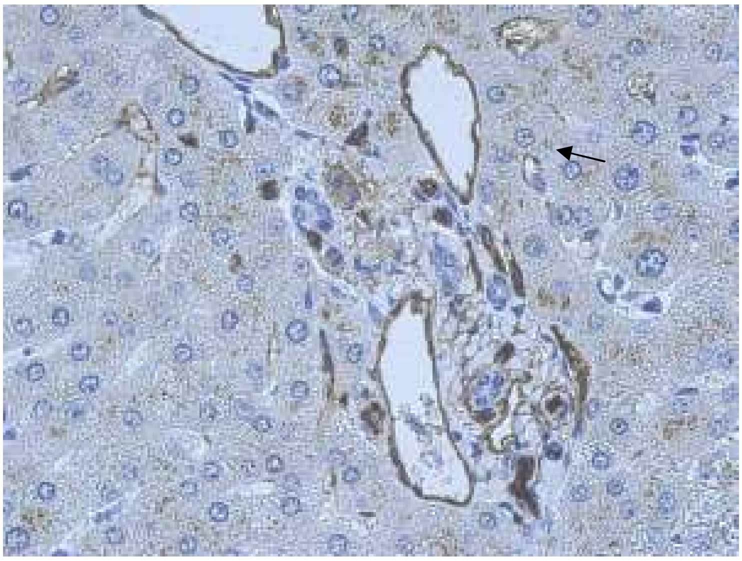


Figure 7b) Immunohistochemistry using CD34 antibody on a section of SCID mouse liver injected with cells cultured from patient with cirrhosis and HCC- Cluster of CD34 positive cells human cells on a background of negatively stained mouse liver cells.

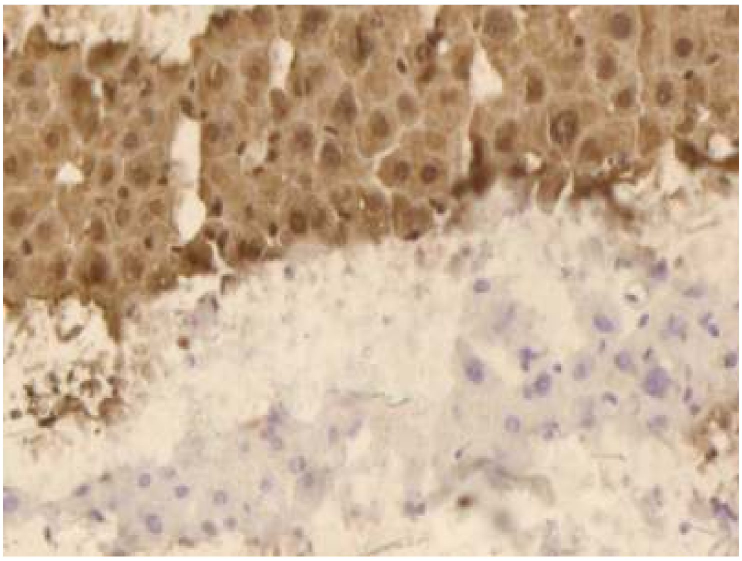
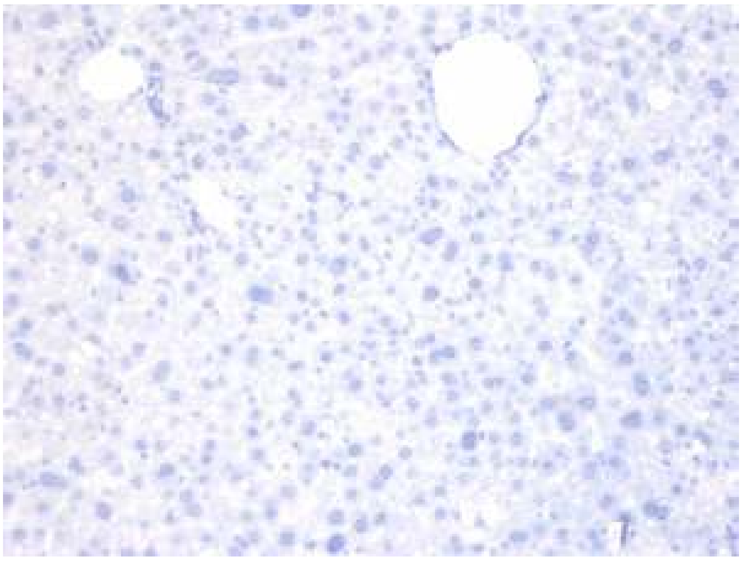
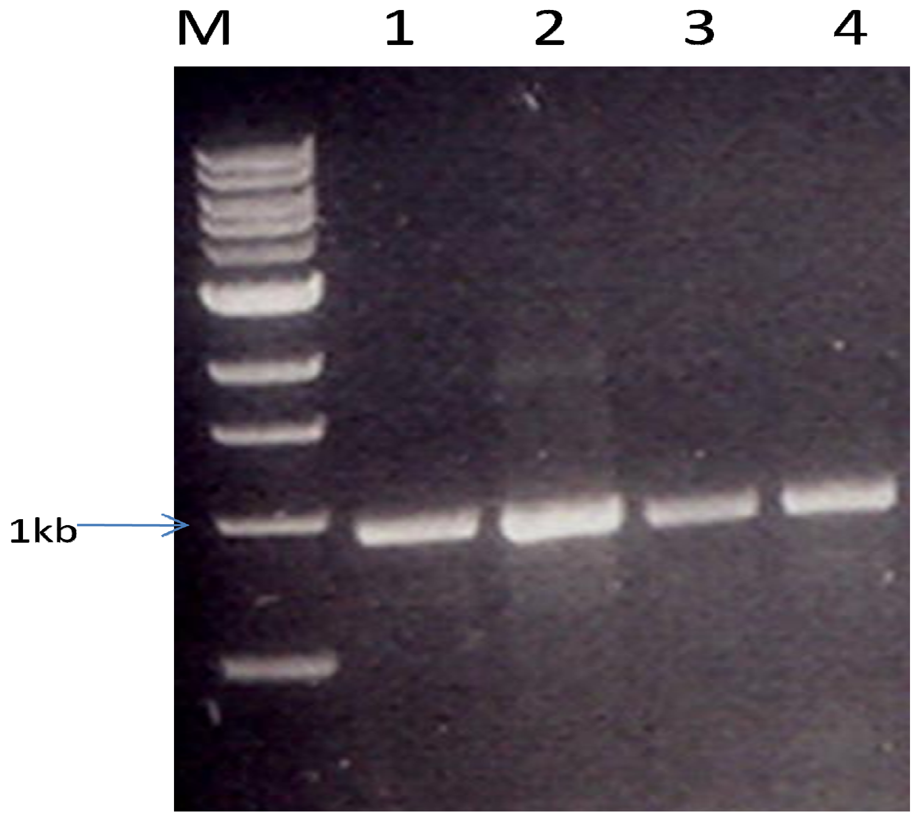


Figure 7c) Immunohistochemistry using CD34 antibody on a section of SCID mouse liver injected with primary human hepatocytes- No positive staining was noted



Molecular analysis of the tissue blocks with dysplastic changes on H&E sections confirmed the source of these changes as human cells (cultured cells from patients with cirrhosis and HCC). The results of human genomic DNA analysis are shown in the figure 8.

Figure 8- Human genomic DNA analysis:



M: 1 Kb marker from NEB

1: Positive control - Human liver tissue

2, 3, and 4: Mouse liver tissue injected with cells cultured from patients with cirrhosis and HCC

Analysis of Kidney sections: Injected cultured cells were only detectable from the kidney sections of mice that received cultured cells from patients with cirrhosis and HCC. These cells demonstrated hepatocyte morphology on H&E staining and expressed cytokeratin 8 and 18 and human MHC class 1 phenotype, but did not express markers of cell proliferation (immunocytochemistry using Ki67 was negative). Following 2 weeks of *in vivo* culture in the renal sub-capsular space no culture cells were detectable in mice that received cells from patients with cirrhosis, but no HCC or primary human hepatocytes. The results are shown in figures 9, 10 and 11.

Figure 9- H&E cryosection of kidney injected with cells from patients with cirrhosis and HCC – Clusters of large hexagonal cells with-in the mouse kidney tissue (arrow).

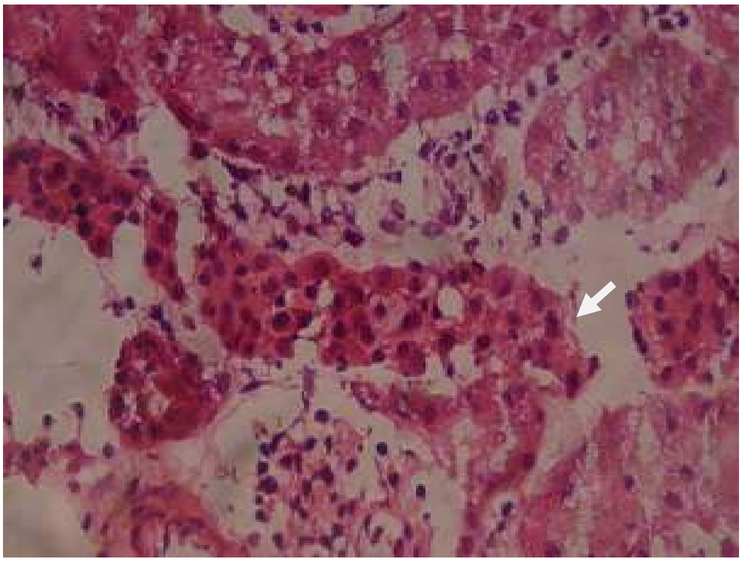


Figure 10a: Immunohistochemistry using antibody against cytokeratin 8 and 18 (Cam 5.2) - Positively stained human liver tissue used as control.

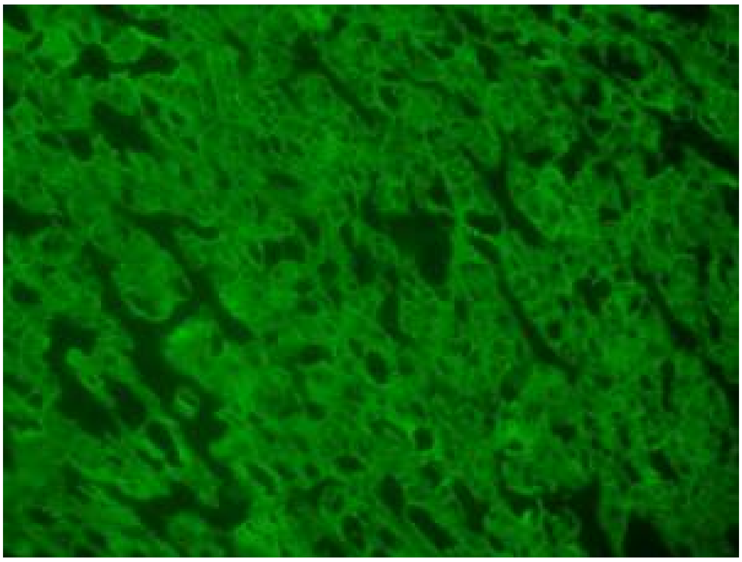


Figure 10b: Immunohistochemistry using Cam 5.2 antibody on a kidney section injected with cells cultured from patient with cirrhosis and HCC- Positively stained cultured cells on a negative mouse kidney background.

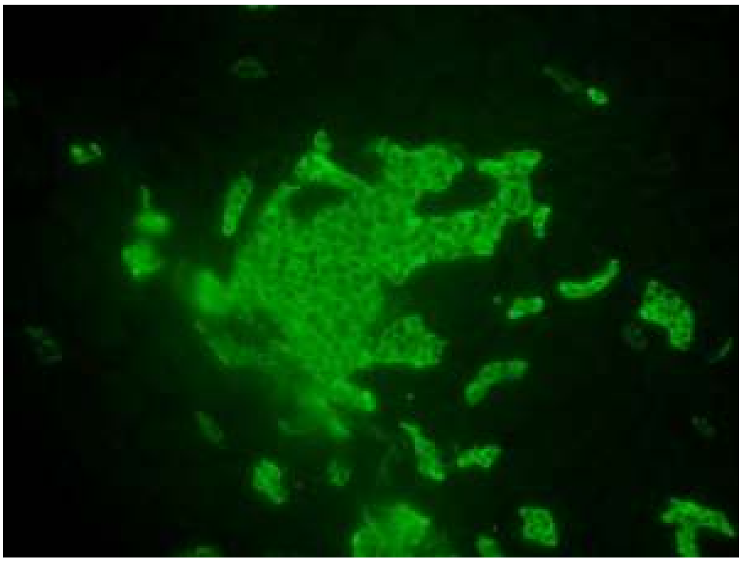


Figure 11a: Immunohistochemistry using antibody against human MHC class-1 antigen - Positively stained human liver tissue used as control.

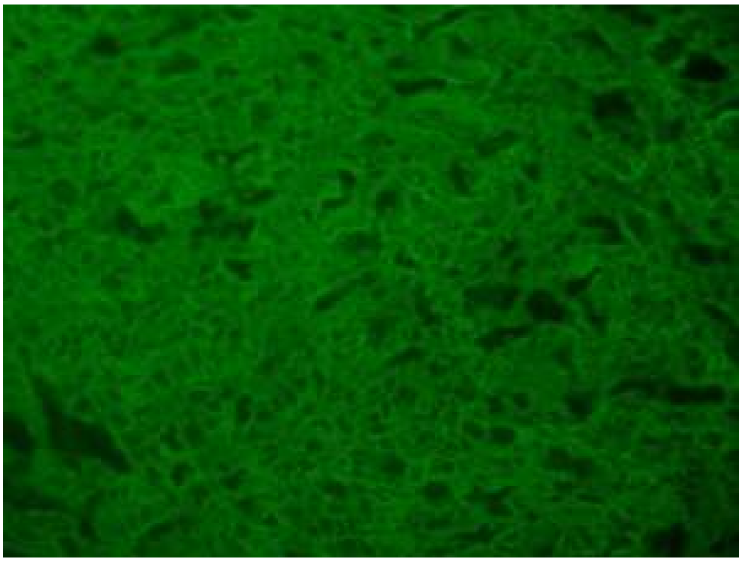
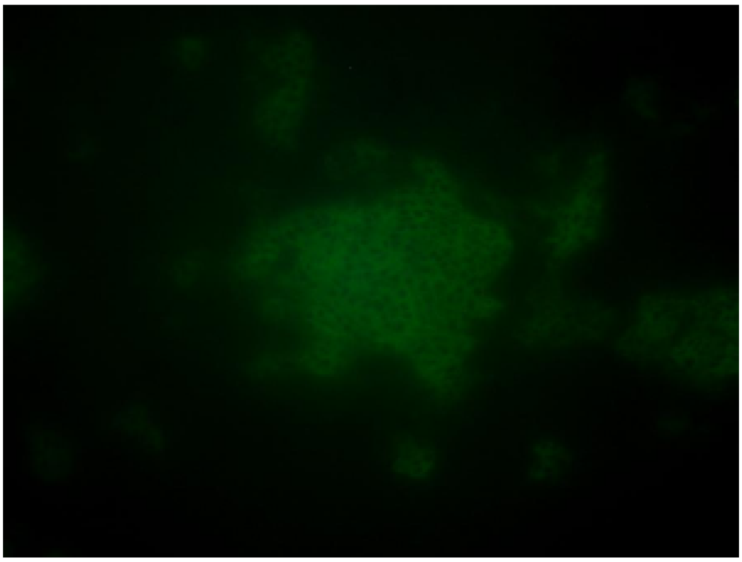


Figure 11b: Immunohistochemistry using MHC class-1 antibody on a kidney section injected with cells cultured from patient with cirrhosis and HCC- Positively stained cultured cells on a negative mouse kidney background.



5.3 Discussion: The *in vivo* biological behaviour of DTC is poorly understood and there are no studies to-date that address this. Experiments describing the methods of detecting DTC only help to identify a sub-group of patients with potentially more aggressive tumour biology. To develop novel cell targeted therapies it is essential to understand the cell pathways associated with survival and propagation of malignant cells. We made an initial attempt to study the *in vivo* behaviour of DTC in HCC and to investigate the potential differences between the cells isolated from patients with cirrhosis and with or without HCC using a murine immunodeficient adoptive transfer model.

The major limitation to the study was the number of cells cultured. To try to overcome this we incorporated the potential advantages of easy localisation of injection of cells into a heterotopic cell transplantation model using a renal sub-capsular site and also provided an optimal physiological matrix using an orthotopic cell transplant model (direct liver injection) in the other. The results of this experiment indicate that the *in vivo* behaviour of the cultured cells from patients with cirrhosis and HCC is different those cultured from patients with cirrhosis and without HCC. Only cultured cells from patients with cirrhosis and HCC were viable following 2 weeks of incubation in the kidney sub-capsular space. These cells were resistant and appeared to have survived despite the small volume injections in a heterotopic environment. Additionally, they may possess pathways that facilitate their escape from the cell mediated lysis induced by natural killer cells of the SCID mouse. Of note, these cells did not express markers of cell proliferation suggesting that they were dormant. Persistence, dormancy and escaping immune lysis are those properties of malignant cells that will result in propagation and recurrence of the tumour despite potentially curative treatments.

The cultured cells from HCC patients also demonstrated features of neoplasia when introduced into a hepatic micro-environment. These changes were confined only to this sub-group of liver injections. A further analysis confirmed the human origin of these neoplastic cells excluding the possibility of cell fusion or of nonspecific changes that could have resulted from cell injections. Although we were not able to demonstrate a macroscopic neoplastic transformation due to the limited cell number, the experiment provided preliminary data regarding the *in vivo* malignant behaviour of DTC from HCC.

5.4 Conclusion: Results from this chapter suggest that the cells isolated from patients with cirrhosis and HCC express features suggestive of a malignant phenotype *in vivo* and thereby differ from those morphologically identical cells isolated from patients with liver cirrhosis and without HCC.

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CHAPTER 6: CLINICAL SIGNIFICANCE OF DISSEMINATED TUMOUR CELLS IN HCC

6.1 Introduction: Hepatocellular carcinoma recurrence is an important cause of death after liver transplantation or liver resection. A pre-operative risk stratification would allow more appropriate allocation of limited cadaveric grafts to lower risk cases, or to identify patients who may benefit from adjuvant therapies. Several tumour related factors have been identified to stratify the risk of recurrence, but these tumour characteristics are not available at pre-operative staging at present. However, this may change as our understanding of tumour behavior increases.

Few studies have focused on identifying surrogate markers of aggressive tumours in blood and bone marrow samples and their methodology has lacked specificity (Sutcliffe 2005). In the previous chapters we demonstrated that hepatocyte-like cells can be isolated from the blood samples of patients with cirrhosis and HCC and that these disseminated cells expressed malignant features both *in vivo* and *in vitro*.

The aim of this chapter was:

1) To evaluate the clinical significance of these disseminated cells detected in patients with cirrhosis and hepatocellular carcinoma

2) To identify any relevant patient or tumour related factors that could result in their dissemination, malignant behavior or establishing metastases.

6.2 Patients and methods: Participants recruited from clinics and wards following a written consent were allocated into three groups: patients with a clinical diagnosis of cirrhosis and HCC, patients with well compensated cirrhosis and without HCC and normal healthy volunteers.

Venous blood was collected and processed according to the protocol described in chapter 2. The presence of DTC in patients with cirrhosis and HCC was confirmed by immunocytochemistry analysis (CK 8&18, Hep Par-1, PCEA, ATP 7b, human albumin, Glycogen, CD133, CD34 and CD90) of the isolated *in vitro* cell colonies.

To facilitate statistical analysis, patients with cirrhosis and HCC were further divided into 3 sub-groups: Group A: robust cell growth (n=6); Group B: unsustained cell growth (n=15); and Group C: no cell growth (n=23), Group B was either amalgamated with Group A or completely excluded.

Data was analysed using SPSS version 17. Descriptive statistics were presented as percentages or median values. The occurrence of DTC was correlated with clinical, radiological and histological parameters. Nonparametric Mann–Whitney *U* test and $\chi 2$ test were used to analyse the differences between the study groups. The survival data was analysed by log rank test and results were plotted using Kaplan Meier survival curves. A p-value of <0.05 was considered significant for all the tests.

6.3 Results:

One hundred and fourteen venous blood samples were obtained from 44 patients with cirrhosis and HCC, 50 patients with cirrhosis and without HCC and 20 healthy volunteers. The relevant clinical parameters of the recruited patients are shown in table below.

	Patients with cirrhosis and HCC (n=44)	Patients with liver cirrhosis and without HCC (n=50)	Healthy volunteers (n=20)
Age (years)	Median 61 (Range 26-82 years)	Median 52(Range 19-67 years)	Median 32(Range 21-45 years)
Sex(M:F)	36:8	35:15	6:14
Hepatitis B	7	3	
Hepatitis C	13	7	
Alcohol	20	22	
MELD	10 (Range 6-20)	14 (Range 7-34)	
Child-Pugh	6 (Range 5-10)	8 (Range 5-13)	

Clinical data of patients with Hepatocellular carcinoma (n=44):

19 patients received potentially curative treatments in the form of liver resection or orthotopic liver transplantation. The median tumour size on radiological staging was 3.3cm (Range 1.2-9cm) and nine patients had multifocal disease. Twenty nine patients (59%) received trans-arterial chemoembolization (TACE) or radio-frequency ablation (RFA), and of these nine (18%) were palliative therapies. Twenty one (42%) had normal serum alpha-feto protein (AFP).

Analysis of variables between sub-groups of patients with HCC: This analysis revealed no significant risk factors that could result in the dissemination of tumour cells into the blood stream. The results were shown in the following tables.

Table 1: Comparative analysis: Group A (Robust cell growth-n=6) +Group B (unsustained cell growth-n=15) vs. Group C (no cell growth-n=23)

Parameter	**P value**
Sex	0.8965
Hepatitis viral infection	0.5156
Tumour size > 5cm (radiology)	0.0719
Number of nodules > 3(radiology)	0.5696
Liver biopsy (prior to blood sampling)	0.1133
TACE (prior to blood sampling)	0.6103
RFA (prior to blood sampling)	0.4105
Micro vascular invasion	0.6210
Degree of tumour differentiation	0.710

Table 2: Comparative analysis: Group A (n=6) vs. Group C (n=23)

Parameter	**P value**
Sex (Female)	0.79
Hepatitis viral infection	0.61
Tumour size > 5cm	0.09
Number of nodules > 3(radiology)	0.62
Liver biopsy (prior to blood sampling)	0.18
TACE (prior to blood sampling)	0.74
RFA (prior to blood sampling)	0.48
Micro vascular invasion	0.82
Degree of tumour differentiation	0.55

Survival data: The clinical follow-up for the 3 sub-groups was last updated in December 2012. The analysis was limited by sample size, the median survivals for Group A, B and C were 13 months (Range 5-34 months), 34 months (Range 2-66 months) and 49 months (Range - 1-79 months) respectively. Kaplan-Meier survival curves showed a trend towards reduced survival in those with DTC, however, this was not statistically significant (P=0.09). The different sub-group analysis is shown in figures 1&2

Figure 1: Survival analysis: Group A (n=6) + Group B (n=15) vs. Group C (n=23)

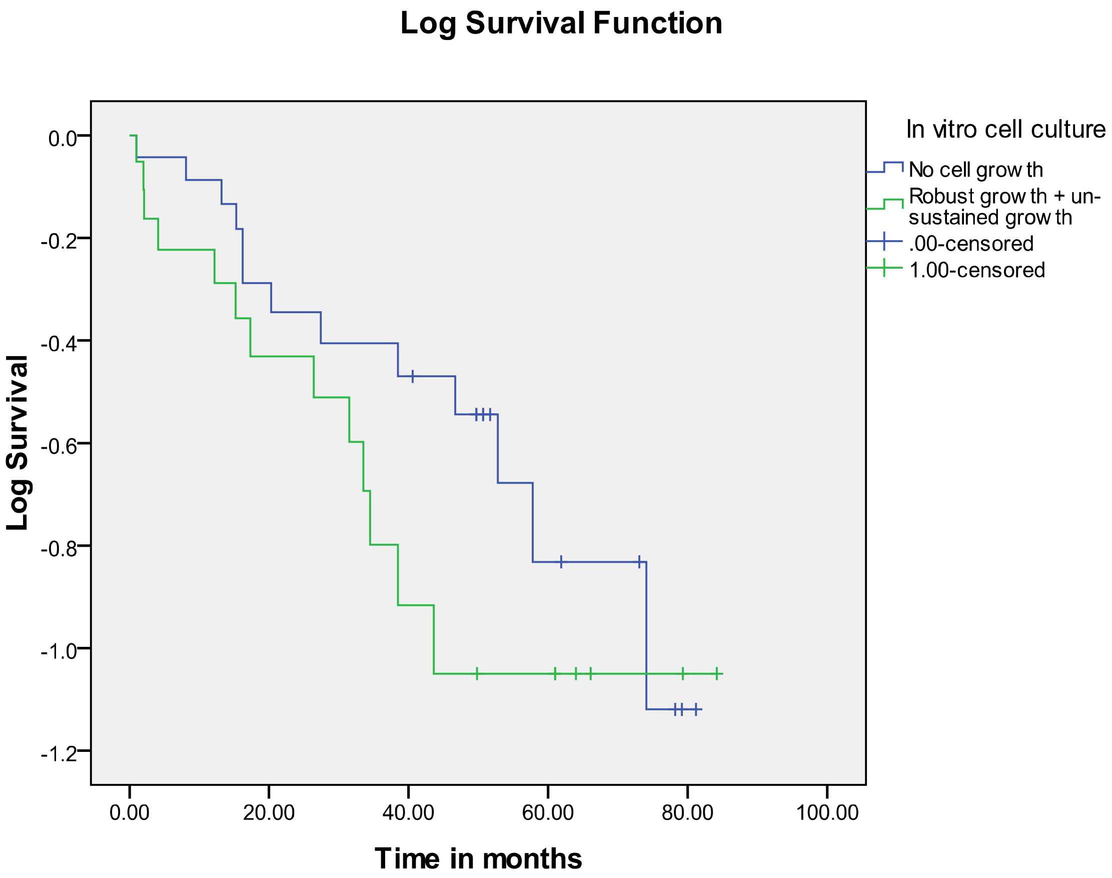
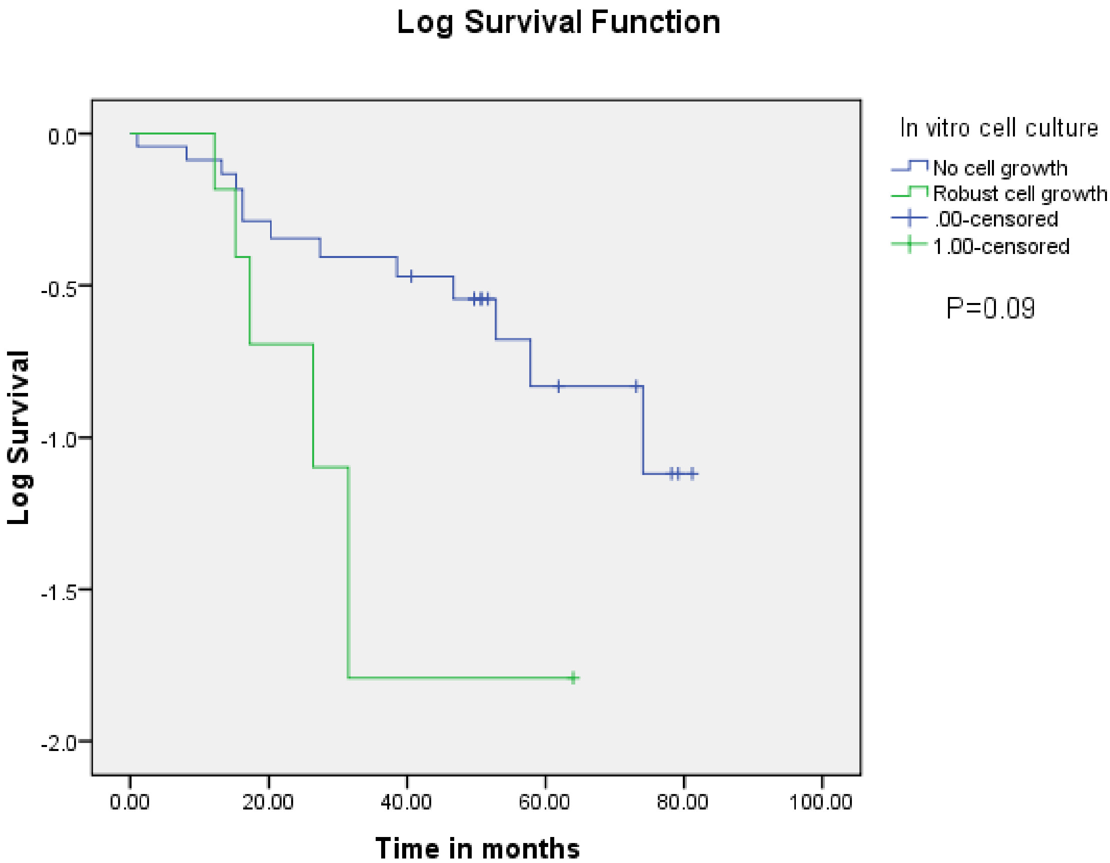


Figure 2: Survival analysis: Group A (n=6) vs. Group C (n=23)



Clinical outcome of patients with cirrhosis: Cell colonies isolated from 3 patients with liver cirrhosis expressed features similar to human hepatocytes, however, immunocytochemical and molecular analysis of these cells showed that they did not have a malignant phenotype. At the end of our clinical follow-up none of these patients developed clinical or radiological evidence of HCC. A trend towards high MELD score was noted in these 3 patients with cirrhosis, but no HCC in whom hepatocyte-like cells were isolated. The scores were 17, 26 and 34 respectively.

6.3 Discussion:

HCC is the sixth most common malignancy worldwide, and the third most common cause of cancer related deaths. At present, curative treatments are offered only to a selected group of patients based on radiological evaluation of tumour bulk and preserved liver function. The reported tumour recurrence rate of up to 25% despite the application of strict tumour criteria highlights the need to improve upon the current staging system.

Current radiologic techniques understage a significant proportion of cases and fail to detect microscopic extrahepatic disease (Mazzaferro 2008). HCC is an aggressive malignancy, studies focussing on the evaluation of microscopic disseminated disease (DTC) and understanding their tumour biology may provide information about risk and patterns of recurrence and aid in development of targeted molecular treatments.

There is growing evidence supporting the use of biological tumour parameters in combination with tumour morphology to improve staging and also develop new treatment modalities. Disseminated tumour cells represent the primary tumour and analysis of these cells can provide in-depth biological and molecular information of the primary cancer. The inclusion of DTC status detected in the blood or bone marrow samples in the staging, risk stratification and therapeutic monitoring of breast cancer patients has been investigated by Pantel et al (Pantel 2009). The 6th edition of the American Joint Committee on Cancer Staging Manual has amended the breast cancer staging by including isolated tumour cells. Microscopic tumour deposits (≤ 0.2 mm) detected by immunocytochemical or molecular techniques are identified as pN0 (i+) where i+ indicates micrometastatic disease.

In a pooled analysis evaluating 4,703 patients with breast cancer, Braun et al. have reported a significantly decreased overall survival in patients with detectable DTC. The presence of DTC was significantly associated with higher tumour stage and was an independent predictor of poor outcome in breast cancer (Braun 2005).

The significance of DTC in HCC and their role in tumour recurrence is still under active investigation. Studies that were designed to detect DTC in blood or bone marrow samples failed to evaluate their molecular and biological properties due to the very limited cell numbers (Sutcliffe 2005; Kienle, 2000; Wong, 1997). The frequency of DTC is 1-2 cells per 1 million bone marrow mononuclear cells and to understand their properties it is essential to increase the cell number by developing a suitable *in vitro* culture protocol.

This study represents the first attempt to develop an *in vitro* protocol to culture and characterize DTC in HCC and further study the *in vivo* behavior using an animal model. The results from chapter 3 show that DTC can be cultured using a hepatocyte favouring cell medium and these cells are similar to human hepatocytes in morphology and function. Additionally they express a stem cell phenotype and aggressive cancer genes which are associated with poor outcomes. The *in vivo* experiment indicated that DTC have an ability to survive by escaping the host cell mediated immune response and induce microscopic features of neoplasia. The clinical data also showed that patients with detectable microscopic disease have a trend towards reduced survival.

Our data indicates that a sub-group of patients with aggressive tumour biology have DTC that can be isolated from a simple venous blood sample (20ml). DTC occurrence is not always associated with tumour morphology and therefore routine radiological staging cannot accurately predict their presence. The detection of DTC could potentially identify patients at risk of recurrence and provide a rationale for adjuvant treatments following potentially curative surgical resections.

There is no strong evidence to support survival benefit with the use of adjuvant chemotherapy in HCC (Samuel 2009). HCC tumours generally respond poorly to systemic chemotherapy agents, and drug resistance has been attributed to presence of effective molecular pathways which repair DNA damage induced by chemotherapy agents (Burroughs 2004). Cancer stem cells have also been implicated in HCC chemo-resistance due to their inherent capacity for self-renewal (Fan ST 2009). Evidence from our study shows that DTC are slow growing and have features of cancer stem cells, these properties may confer chemo-resistance.

To improve survival there is a need to develop effective adjuvant treatment modalities that can tackle microscopic disease. Studies to further understand the molecular and immunocytological properties of DTC may help in the development of specific targeted therapies in future. Monitoring of DTC in blood samples could also be used as a tool to measure the effectiveness of systemic adjuvant therapies and potentially test resistance to therapeutic agents *in vitro.*

Our study also showed that circulating hepatocyte-like cells are present in patients with end stage liver disease. The observation that these cells isolated from a small number of patients with high MELD score indicates that severity of liver injury may have an influence on their occurrence. The role of these hepatocyte-like cells in restoring liver mass and their possible contribution to the development of HCC in the presence of chronic liver injury needs to be addressed by future studies.

6.4 Conclusions:

The results from this chapter suggest that presence of DTC is associated with aggressive tumour phenotype and reduced median survival. Incorporation of tools to assess micrometastatic disease burden into the current staging may improve patient selection for potentially curative surgical treatments and also identify a subgroup of patients who may benefit from adjuvant treatment. However, firm conclusions cannot be made due to limited sample size. Larger studies are needed to confirm our findings and expand analyses of the molecular and biological properties of DTC to develop novel treatment modalities that can reduce the microscopic tumour burden.

CHAPTER 7: DISCUSSION

HCC is an aggressive malignancy and potentially curative surgical procedures can only be offered to a small group of carefully selected patients using strict staging criteria. Currently tumour morphology assessed by radiological imaging is the only variable used as a surrogate marker of tumour biology. It is estimated that 25% of HCC are under staged with the current radiological imaging. Microvascular invasion and grade of tumour differentiation are strong predictors of tumour recurrence, however, current practice does not seek this information routinely before implementing curative treatments. A recent study by Mazzaferro et al (2008) showed that 10% of tumours within Milan criteria have microvascular invasion and are poorly differentiated. The study highlighted that tumour morphology on its own is not an accurate predictor of tumour biology but it has been accepted in clinical practise due to lack of robust biological or molecular markers that can predict HCC behaviour. The treatment of HCC is further complicated by the fact that the prognosis of patients who develop tumour recurrence is dismal due to lack of effective systemic therapies.

In an effort to refine the current staging and understand factors related to tumour recurrence some studies have focussed on biological or molecular surrogate markers such as microscopic disseminated disease, plasma albumin mRNA and serum alpha-fetoprotein. To date, however, none of these have been successfully translated into clinical practice.

Disseminated tumour cells detected in the blood or bone marrow have been associated with metastasis, chemoresistance and poor prognosis in several epithelial malignancies. Our previous study developed an effective tool to detect these disseminated cells, however, due to their scarce occurrence it was not possible to study their molecular and biological properties. To understand the role of DTC in progression of HCC or to develop specific targeted treatment modalities the initial step was to increase their cell numbers to facilitate molecular and immunochemical analysis.

This study represents the first attempt to develop a protocol to culture the DTC in patients with chronic liver disease and HCC and study their biological properties. In Chapter 3, venous blood samples from patients with cirrhosis and HCC were cultured with the relevant controls using a hepatocyte favouring culture medium. This technique yielded encouraging results by supporting the development of robust cell colonies in 6 out of 44 patients with HCC, 3 out of 50 patients with liver cirrhosis and none out of 20 normal healthy volunteers. All of these cultured cells co-expressed hepatocellular markers; cytokeratin 8/18 (Cam 5.2), hepatocyte specific antigen (Hep par 1) and polyclonal CEA along with albumin and glycogen and are characteristic of hepatocyte function.

Cancer stem cells (CSC) have been associated with tumour propagation and poor prognosis in HCC. The hepatocyte like cell colonies isolated from our first experiment were further analysed for the presence of stem cell markers. CSC in HCC are also known to share a variety of markers with haematopoietic stem cells causing conflicting opinions regarding their origin, however, to-date there are no clear conclusions. While some studies favour the bone marrow as their source, others support the hepatic origin of these cells. We studied the relationship between cancer stem cells and DTC isolated in our study using well described markers (CD34, CD133, and CD90) and attempted to locate their source.

We observed that whilst CD34 was widely expressed, CD133 and CD 90 expression was only confined to a very few cells that were derived from patients with cirrhosis and HCC. CD133 and CD90 are shown to be expressed by malignant hepatoma cells. Experience from histological analysis of primary human HCC as well as human hepatoma cell lines demonstrated a positive correlation between their expression and biological tumour aggressiveness. The low expression of cancer stem markers CD133 and CD90 by our cultured cells derived from patients with HCC is consistent with previously published data on primary HCC.

The question regarding the origin of DTC was addressed using a microarray analysis of high gene expression in cells cultured from patients with HCC, the HCC cell line HepG2 and primary human hepatocytes, compared with purified $CD34^{+ve}$ bone marrow cells. The analysis showed that DTC cultured from patients with cirrhosis and HCC expressed hepatocellular markers which excluded their being bone marrow stem cells. These results suggested that DTC express markers of cancer stem cells and their source of origin is the liver.

In our study a very small sub-group of controls with liver cirrhosis (3/50) also had circulating cells. These cells expressed markers of hepatocellular lineage and CD 34, but not CD133 or CD90. To investigate the differences between the circulating cells isolated from patients with cirrhosis and HCC and those without HCC we used the 3 gene expression profile that was described by Llovet et al to discriminate HCC from dysplastic or cirrhotic nodules. The qPCR analysis revealed an increased expression of glypican 3 and surviving, but a reduced expression of LYVE1 in the cells cultured from patients with HCC when compared with cells obtained from patients with cirrhosis, but no HCC. These results were consistent with those published by Llovet et al (2006). We also noted that the expression of TGF-alpha and p53 was consistently increased in cells derived from patients with cirrhosis and HCC when compared with those cultured from patients with only cirrhosis. These data indicated that a malignant phenotype is expressed only by circulating cells cultured from patients with cirrhosis and HCC.

A further microarray analysis of cancer gene expression by the DTC revealed an over expression of 18 genes that are associated with poor prognosis in HCC and 43 genes that are associated with poor outcomes in non-HCC solid organ tumours.

The *in vitro* isolation and analysis of DTC from blood samples of patients with cirrhosis and HCC showed that a small sub-group of patients can shed microscopic cells that are potentially malignant.

The *in vivo* malignant potential of cultured DTC was tested using a SCID mouse cell transplantation model. A small volume of cultured cells ($2x10^4$) was injected directly into renal sub-capsular space and liver. Following an incubation period of two weeks it was observed that only cells from patients with cirrhosis and HCC survived in the renal sub-capsule and were detectable on H&E as well as immunohistochemistry staining. Interestingly the histology of liver sections injected with cells from HCC showed features of neoplasia while the control liver sections had normal architecture. The expression of human CD34 and human genomic DNA by the dysplastic liver sections excluded the possibility cell fusion or non-specific changes.

These results showed that cells cultured from patients with HCC express molecular and biological properties that are different when compared to those isolated more infrequently from cirrhotic controls, reflecting their cancer lineage.

Taking into consideration the small sample size, the analysis of clinical data suggested that the presence of DTC is not influenced by morphological parameters of the tumour or any pre-operative interventions. The patients with isolated DTC had a trend towards worse clinical outcomes although the results were not statistically significant.

Large studies from breast cancer research groups have identified the presence of DTC as an independent predictor of poor survival. The association between the presence of DTC and metastatic relapse of breast cancer has also been shown by published studies (Braun 2005, Pantel 2008). Breast cancer metastasis models demonstrated that microscopic metastatic cancer cells detach early from primary tumours and are released into blood and BM. These microscopic cells are not detected by routine pre-operative radiological staging investigations but peripheral blood analysis could provide valuable information on the tumour dissemination. A pooled analysis from 9 European centres including more than 4000 patients showed that 30% of women with primary breast cancer have DTCs. A 10-year survival analysis of breast cancer patients with DTCs revealed significantly decreased overall survival when compared to those with no DTCs (Braun 2005).

The success of HCC treatment depends on robust pre-operative staging to identify extra-hepatic disease and to plan down-staging or adjuvant chemotherapy. Early spread of microscopic tumour cells is undetected even by high-resolution imaging technologies which prevents any early intervention to improve outcomes of potentially curative treatments. DTCs can escape the host immune system in a dormant state and have the potential to develop into overt metastases when activated by internal/external signals.

Our study shows that occult disseminated tumour cells are detectable in peripheral blood of a sub-group of HCC patients with aggressive phenotype and their presence cannot be determined by the pre-operative staging investigations. Further, the biology of HCC cannot be assessed accurately with tumour morphology alone. The current selection criteria could be improved by inclusion of additional markers which predict the tumour biology. DTCs represent the parent tumour and their isolation and characterisation could shed light on subsequent tumour behaviour. We demonstrated that DTC in HCC could be isolated from simple peripheral venous blood samples and expanded by *in vitro* culture for further characterisation. The detection of microscopic disease may offer further insight into the HCC and improve the utilisation of current surgical procedures (especially liver transplantation) and clinical outcomes. An in-depth knowledge of the molecular and biological properties of DTC could lead to the development of specific targeted therapies that can potentially down stage a tumour or provide adjuvant treatments following surgery.

Further studies to characterise circulating hepatocyte-like cells in patient with cirrhosis may help in understanding the underlying mechanisms of liver regeneration in chronic liver injury and the role of stem cells and HCC transformation pathways.

7. Future work:

Objective	Methodology
Develop an optimal medium to increase the cell number	1) Supplement the current medium with more growth factors/insulin/dexamethasone 2) Use of Kubota's medium that favours growth of hepatoblasts/neonatal liver cells (Kubota et al_Stem Cells. 2007 Sep;25(9):2339-49) Or Develop an immortalised cell line by microinjection with T antigen DNA(Pantel et al; Journal of the National Cancer Institute, Vol. 87, No. 15, August 2, 1995)
Assess the validity of alternative markers of micrometastases	Immunocytochemical analysis using antibody markers e.g. pCEA, TTF-1, cystatin B
Application of new methods of detecting microscopic tumour cells	Use of multimarker RT-PCR with a panel of tumor specific mRNA markers, Enzyme linked immunospot technology (ELISPOT), Automated detection (e.g. Automated microscopy, laser scanning cytometer)
Study the interactions between disseminated tumour cells and hepatic endothelium to understand the pathways of tumour recurrence in liver	1. Compare the effects of serum on tumour cell adhesion using larger number of patients with and without post-transplant HCC recurrence 2. Identify specific serum factors that modify tumour cell adhesion to endothelium
Improve SCID mouse animal model	1.Induce growth of injected cells by performing a hepatectomy 2. Use of radio labeled markers to trace injected cells.

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APPENDICES

Appendix I

Classification of hepatocellular carcinoma

a: Okuda classification

	Negative	Positive
Tumour size Ascites Serum albumin Bilirubin	<50% of liver Absent >3 g/dl <3 mg/dl	>50% of liver Present <3 g/dl >3 mg/dl

Okuda I: No positive factor; Okuda II: 1 or 2 positive factors; Okuda III: 3 or 4 positive factors

b:French classification

	0	1	2	3
Karnofsky index (%)	≥80			<80
Bilirubin (µmol/l)	<50			≤50
Alkaline phosphatase (MNL)	<2		≥2	
Alpha-1 fetoprotein (µg/l)	<35		≥35	
Portal obstruction (US)	No	Yes		

Karnofsky index >80% = complete patient autonomy; MNL = maximum normal limit; US = ultrasound. Group A (low risk): 0 point; group B (intermediate risk): 1-5 points; group C (high risk): ≥6 points c: Chinese University Prognostic Index(CUPI) classification

Variable	Score
TNM classification	
I and II	-3
III A and III B	-1
IV A and IV B	0
Asymptomatic at diagnosis	-4
Ascites	3
AFP ≥500 ng/ml	2
Bilirubin (μmol/l)	
<34	0
34-51	3
>51	4
Alkaline phosphatase ≥200 IU/l	3

Score ≤1: low risk; score 2-7: intermediate risk; score ≥8: high risk. Risk of death within 3 months: >70% (high risk); 30 to 70% (intermediate risk); <30% (low risk). AFP = alpha-1 fetoprotein.

d: Cancer of the Liver Italian Program (CLIP) classification:

Variable	Points
Child-Pugh	
A	0
B	1
C	1
Tumor morphology	
Uninodular and extension <50%	0
Multinodular and extension ≤50%	1
Diffuse (massive) or extension >50%	2
Alpha-1 fetoprotein	
<400 ng/ml	0
≥400 ng/ml	1
Thrombosis of the portal vein	
No	0
Yes	1

Clip classification: 0 to 6 points

e: Barcelona Clinic Liver Cancer (BCLC) classification of Hepatocellular carcinoma

Stage	PST	Tumour stage	Okuda	Liver function	Remarks
Stage A: early HCC					*Stage A and B* All criteria should be fulfilled
A1	0	Single	I	No CRPH + bil N	
A2	0	Single	I	CRPH + bil. N	
A3	0	Single	I	CRPH + bil.↑	
A4	0	3 tumours ≤3 cm	I-II	Child A- B	
Stage B: intermediate HCC	0	Multinodular	I-II	Child A-B	
Stage C: advanced HCC	1-2	Vascular invasion/ extrahepatic spread	I-II	Child A-B	*Stage C* At least one criterion: PST 1-2 or vascular invasion/ extra-hepatic spread
Stage D: end-stage HCC	3-4	Any	III	Child C	*Stage D* At least one criterion: PST 3-4 or Okuda III/Child C

PST, performance status; CRPH -clinically relevant portal hypertension; bil- bilirubin; N- normal.

f: Japan Integrated Staging (JIS) scoring system

Variables	Scores			
	0	1	2	3
Child-Pugh grade	A	B	B	
TNM stage by LCSGJ	I	II	III	IV

The JIS score is calculated by summing the scores for the Child-Pugh classification (*C-P grade*) and the TNM tumour stage according to the Liver Cancer Study Group of Japan criteria

TNM stage by Liver Cancer Study Group of Japan criteria (LCSGJ)

Factors	I. Single	II. Size <2cm	III. No vessel invasion
T1		Fulfilling three factors	
T2		Fulfilling two factors	
T3		Fulfilling one factor	
T4		Fulfilling 0 factors	
Stage I		T1 N0 M0	
Stage II		T2 N0 M0	
Stage III		T3 N0 M0	
Stage IV-A		T4 N0 M0 or T1-T4N+M0	
Stage IV-B		T1-T4, N0 or N1, M+	

Appendix II

The Child- Pugh classification for severity of liver disease

Score	1	2	3
Bilirubin (µmol/l)	<34	34-50	>50
Albumin (g/l)	>35	28-35	<28
PT INR	<1.7	1.71-2.3	>2.3
Encephalopathy	none	mild	marked
Ascites	none	mild	marked

<7 = Class A; 7-9 = Class B; >9 = Class C

Appendix III

Common antibody stains used to diagnose hepatocellular carcinoma*

Antibody	Results of Immunostains (%) (overall, range)
α-fetoprotein (AFP)	50 (12–75)
Polyclonal carcinoembryonic antigen (pCEA)	70 (60–95)
CD10	68 (60–95)
HepPar1	80–100 (40–100)
CAM 5.2	>90 (90–95)
CK 8	Usually positive
CK 18	Usually positive
AE1/AE3	20 (5–33)
CK 7	15 (0–25)
CK 19	10 (0–20)
CK 20	20 (10–30)
CD 34	Usually positive

* Wee A; Diagnostic utility of Immunohistochemistry in hepatocellular carcinoma, its variants and their mimics; Appl Immunohistochem Mol Morphol. 2006 Sep; 14(3):266-7

Appendix IV

Information sheets

a: Information to HCC patients participating in micrometastases study

This sheet contains information about what to expect if you are taking part in the study. Your participation is entirely voluntary. Please keep this sheet for future reference.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at the questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW. If you have any concerns or questions which are not covered here, please contact Mr Narendra Battula (Tel: 07743846045) for further information.

What is this study?

The aim of this study is to try to improve the treatment of patients with liver cancer. by looking at peripheral blood samples, it may be possible to detect cancer cells earlier, before they are visible on scans, so that treatment can be improved. In addition to the special tests that you will have had already, we plan to perform another blood test. These samples will be analysed to see if there are any sign of liver cells.

If I agree to enter the study, can I withdraw subsequently?

Yes. If you consent to take part in the study, you are under no obligation to continue and you are free to withdraw at any time without giving a reason. Your future care and treatment will not be affected if you decide to withdraw from the study.

Is the procedure painful?

Blood samples will be collected at the time of your routine blood tests, it is safe and there are no long term side effects.

Will the study affect my treatment?

No. The treatment you receive will be exactly the same whether you decide to enter the study or not. The results of the study will not benefit you directly, but hopefully it will improve our understanding of liver cancer, and may help the treatment of others in the future.

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b: Information to control patients participating in micrometastases study

This sheet contains information about what to expect if you are taking part in the study. Your participation is entirely voluntary. Please keep this sheet for future reference.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at the questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW. If you have any concerns or questions which are not covered here, please contact Mr Narendra Battula (Tel: 07743846045) for further information.

What is this study?

The aim of this study is to try to improve the treatment of patients with liver cancer. by looking at peripheral blood samples, it may be possible to detect cancer cells earlier, before they are visible on scans, so that treatment can be improved. We also need to analyse blood samples from patients without cancer such as you for comparison. In addition to the special tests that you will have had already, we plan to perform another blood test. These samples will be analysed to see if there are any sign of liver cells.

If I agree to enter the study, can I withdraw subsequently?

Yes. If you consent to take part in the study, you are under no obligation to continue and you are free to withdraw at any time without giving a reason. Your future care and treatment will not be affected if you decide to withdraw from the study.

Is the procedure painful?

Blood samples will be collected at the time of your routine blood tests, it is safe and there are no long term side effects.

Will the study affect my treatment?

No. The treatment you receive will be exactly the same whether you decide to enter the study or not. The results of the study will not benefit you directly, but hopefully it will improve our understanding of liver cancer, and may help the treatment of others in the future.

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Appendix V

Consent form

Department	**Institute of Liver Studies**
Title of Study	**Micrometastases in hepatocellular carcinoma**
Investigator's Name	**Mr Narendra Battula**

To be completed by the subject/patient/parent/guardian *(delete as necessary)*

1. Have you read the information sheet about this study? YES/NO
2. Have you had an opportunity to ask questions and discuss this study? YES/NO
3. Have you received satisfactory answers to all your questions? YES/NO
4. Have you received enough information about this study? YES/NO
5. Do you understand that you are free to withdraw from this study at any time without giving a reason for withdrawing without affecting your future relationship with the medical or nursing care YES/NO
6. Do you agree to take part in this study? YES/NO

Signed: ________________________________Date _____________________

Name in Block letters ____

Signature of investigator ____

Please note:

7. For persons under 18 years of age the consent of the parents or guardians must be obtained and the assent of the child/young person should be obtained to the degree possible dependent on the age of the child/young person.
8. In some studies witnessed consent may be appropriate.
9. One copy of the signed consent form will be given to the patient, one will be kept by the investigator and one will be kept for the notes.

The consent form must be signed by the actual investigator concerned with the project after having spoken to the subject to explain the project and after having answered his or her questions about the project.

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Appendix VI

Alphabetical list of Reagents

a: Reagents

Acetone	Sigma, UK
Acetic acid	Sigma, UK
Accutase	Sigma, UK
Alpha-Minimum Essential Medium	Invitrogen, UK
Antibiotic & Antifungal	Invitrogen, UK
Anti-human albumin-FITC conjugated	Dako, UK (working dilution 1:50)
Anti-human albumin	Sigma UK (unconjugated mouse monoclonal antibody; woking dilution 1:250)
Anti-mouse fluorescent kit	Vecta, UK
ATP 7b (unconjugated mouse monoclonal) antibody	Abcam, UK (working dilution 1:500)
Human bone marrow stromal cells (hMSC)	Donation by Tulane University, USA
CD68 - unconjugated mouse monoclonal antibody	Sigma UK (working dilution 1:500)
CD34 - unconjugated mouse monoclonal antibody	Abcam, UK (working dilution 1:250)
CD133 - unconjugated mouse monoclonal antibody	Sigma, UK (working dilution 1:250)
CD 90- unconjugated mouse monoclonal	Dako, UK (antibody working dilution 1:100)
Collagen (Bornstein and Traub Type I calf skin)	Sigma, UK (0.3mg/ml in 10mM acetic acid)
Collagenase	Sigma,UK (Type 4)
Collagenase	Sigma, UK (Type 1a)

Cam 5.2 (Cytokeratin 8 & 18) antibody	Becton-Dickinson, UK (readymade to use)
Dimethylsulphoxide	Life Technologies, UK
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, UK
Endothelial cell growth medium MV2	Promo cell, UK
Ethanol (75%)	Commercial suppliers, King's College Hospital
Fetal Calf Serum (FCS)	Invitrogen, UK
Fluorescent secondary antibody	Vecta Kit
GeneChip Hybridization, Wash, and Stain Kit	Affymetrix, USA
Haematoxylin, Carazzi's	Sigma, UK
Haematoxylin, Mayer's (Lillie's modification)	Dako, UK [Contains haematoxylin (5 g/l), aluminum ammonium sulphate (45 g/l), glycerine (30%), sodium iodate (0.2 g/l), pH 2.4]
Human hepatoma cell line (Hep G2)	ECACC, UK
Hep Par-1 (mouse anti-human hepatocyte monoclonal antibody clone OCH1E5)	Dako, UK (working dilution 1:500)
Heparin	CP Pharmaceuticals UK
Histopaque-1077	Sigma, UK (polysucrose, 5.7g/dl; sodium diatrizoate, 9.0g/dl)
Human Hepatocytes	Donation from hepatocyte transplantation unit, King's College Hospital, UK
Human colon cancer cell line CaCo-2	ECAAC,UK
Human gene 1.0 ST array	Affymetrix, USA
Hypnorm	(0.315mg/ml Fentanyl citrate + 10 mg/ml Fluanisone)
Hydrogen peroxide (H2O2)	3% in PBS

Isopropropanol	Sigma, UK
Ki-67 (unconjugated rabbit polyclonal)	Abcam, UK (working dilution 1:500
L-Glutamine	Invitrogen, UK
Liquid nitrogen	Commercial suppliers, King's College Hospital.
Midazolam	Commercial suppliers, King's College Hospital
MHC class-1 (unconjugated mouse monoclonal)	Dako, UK (working dilution 1:250)
Novacastra Ready-to-Use peroxidase detection systems	Novacastra laboratories, UK
NuGEN Pico WT- Ovation labelling kit	NuGEN Inc, USA
Percoll density gradient medium	Sigma, UK
Periodic acid Solution 0.5% (Periodic acid -0.5 g in 100ml of distilled water)	Sigma, UK
Phosphate-buffered saline (PBS)	Dako, UK (Contains 20mM sodium phosphate, 150mM sodium chloride, pH 7.0)
Propidium iodide (PI)	Vecta, UK
Polyclonal CEA (unconjugated rabbit polyclonal)	Dako, UK (working dilution 1:250)
Real time PCR system(7900 HT)	Applied Biosystems Inc, USA
SCID mice (6 week old, Male)	Charles River UK Ltd
Schiff Reagent	Sigma, UK
Anti-Smooth muscle actin	Dako, UK (working dilution 1:250)
TaqMan Gene expression master mix	Applied Biosystems Inc, USA
Trypsin EDTA	Invitrogen, UK
Vectastain Streptavidine/Peroxidise kit	Vecta, UK
Trizol reagent	Invitrogen, UK

Tryphan blue	Invitrogen, UK
Xylene	Sigma, UK

b) Media

Cell culture medium- Disseminated cells	500ml of Alpha minimum essential medium enriched with 20% fetal calf serum (Invitrogen, UK), 1% L-Glutamine (Invitrogen) and 1% antibiotic and antifungal reagents (Invitrogen, UK).
Cell culture medium- endothelial	500 ml of Endothelial cell growth medium MV2 (promo cell) supplemented with 10% fetal calf serum, 10% endothelial growth factor and 1% penicillin and streptomycin.
Cell culture medium- hepatocytes	500 ml of William's E medium (Sigma) enriched with 1% penicillin and streptomycin, 10% fetal calf serum, 10MmHepes and 1% L-Glutamine.
Cell culture medium- colon cancer cell lines	500 ml of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FCS, 1% penicillin and streptomycin and 1% non essential amino acids.

All of the above prepared medium were stored at 4˚C and used within 4 weeks.

DECLARATION

The research work undertaken for this Thesis was conducted entirely by the Author Narendra Battula after receiving appropriate training in laboratory techniques. Professor Nigel Heaton and Dr Varuna Aluvihare acted as Clinical Supervisors for the project. Postdoctoral scientists (Drs Guo cai Huang, Min Zhao, Helen Brereton and Siamak Salehi) provided training in laboratory methodology and were available on a day-to-day basis for support and advice. Ethical approval for this project was granted by King's College Hospital Research and Ethics Committee on 12th June 2001, and all patients were fully informed of the nature of the research and provided written consent. The research work was carried out in the Institute of Liver Studies, King's College Hospital, London, UK and in the Rayne Institute, Denmark Hill Campus, King's College London, UK.